REMARKS

Claims 1-10 are pending. Claims 1, 5 and 6 are amended herein. Claims 2-4 and 7-10 are cancelled and new claim 11 is added. Accordingly, upon entry of the present amendment, claims 1, 5, 6 and 11 are pending.

Support for the amendments to claims 1, 5 and 6 and new claim 11 can be found throughout the instant specification as filed, and claims 2-4. No new matter is added by way of these amendments.

Amendment and cancellation of the claims here are not to be construed as an acquiescence to any of the rejections/objections made in the instant Office Action or in any previous Office Action, and were done solely to expedite prosecution of the application. Applicants hereby reserve the right to pursue the claims as originally filed, or substantially similar claims in one or more subsequent patent applications.

Claim Objections

Claims 3 and 9 are objected to for the alleged informality of repeating diseases unnecessarily. Applicants note that claims 3 and 9 are cancelled, obviating the objection with respect to these claims. Applicants further note that, solely in the interest of expediting prosecution of the instant application, claim 1 is amended to recite diseases or disorders only once. Accordingly, Applicants request withdrawal of this objection.

Specification

The Office Action objects to the formatting of the specification as filed. Solely in the interest of expediting prosecution of the instant application, Applicants herein amend the instant specification to provide section titles.

Rejection Under 35 U.S.C. § 112, First Paragraph, Enablement

The Office Action rejects claims 1-9 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. According to the Office Action, the specification is "enabling for treatment of most diseases of claims 1-3 and 7-9, with the exception of multiple sclerosis (MS) and

amyolateral sclerosis (ALS) (claim 3), [but] does not reasonably provide enablement for treating and certainly not the prevention of MS or ALS, or the prevention/cure for any of the other diseases of claims 1-3 and 7-9." Applicants respectfully disagree and traverse the rejection for at least the following reasons.

Applicants note that the rejection of claims 2-4 and 7-9 under 35 U.S.C. §112, first paragraph, is rendered moot by the cancellation of those claims. Regarding claim 1, and claims 5 and 6 depending therefrom, solely in the interest of expediting prosecution of the instant invention, claim 1 is amended to require "***treatment of*** diseases or syndromes selected from the group consisting of nitrate-induced tolerance, a disease of the eye, central retinal arterial occlusion, posterior ciliary arterial occlusion, central retinal venous occlusion, optical neuropathy, macular degeneration, diabetes, premature labor, preeclampsia, alopecia, psoriasis, renal syndrome, cystic fibrosis, cancer, age-associated learning and memory disturbance, age-associated memory loss, craniocerebral trauma, post-traumatic craniocerebral trauma, concentration disturbance in a child suffering from learning and memory problems, dementia associated with Lewy bodies; dementia associated with degeneration of the frontal lobes including Pick's syndrome, Parkinson's disease, progressive nuclear palsy, dementia associated with corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jacob dementia, new variant Creutzfeld-Jacob dementia, HIV dementia, schizophrenia associated with dementia and schizophrenia associated with Korsakoff's psychosis in a subject [with] an effective amount of a cGMP-stimulating compound" (***emphasis added***). As cited above, the instant Office Action expressly acknowledges that the specification as filed is "enabling for treatment of most diseases of claims 1-3 and 7-9." However, the Office Action specifically alleges that treatment of two diseases, multiple sclerosis (MS) and amyolateral sclerosis (ALS), is not enabled. Applicants respectfully submit that one of ordinary skill in the art would find the instant specification enabling for treatment of all diseases and syndromes recited in the present claims, including ALS and MS. Indeed, the instant Office Action offers no specific reason for excluding ALS and MS from the list of diseases and syndromes that can be treated *via* administration of the cGMP-stimulating compounds of the invention. As recited in the Office Action,

> [A] [s]pecification disclosure which contains teaching of manner and process of making and using the invention in terms corresponding to the scope to those used in describing and defining subject matter sought to be patented ***must be taken as***

> ***in compliance with the enabling requirement*** of the first paragraph of § 112 ***unless there is reason to doubt the objective truth of statements contained therein which must be relied on for enabling support.*** (***emphasis added***)

In re Marzocchi, 439 F.2d 220

For the instant invention, one of ordinary skill in the art at the time of filing would not have possessed any reason to doubt the objective truth of Applicants' statements regarding the use of cGMP-stimulating compounds in treatment of any of the diseases or syndromes recited in the instant claims, including ALS and MS. As stated in the instant specification in reference to diseases and syndromes treatable with the compounds of the instant invention (expressly including treatment of ALS and MS, *e.g.*, at least at page 21, lines 8-9 of the specification as filed), "the compounds [of the invention] are of interest for all therapies in which an increase in the concentration of cGMP can be assumed to be curative" (page 20, lines 3-5). As was known in the art at the time of filing, ALS and MS are both diseases for which modulation of cGMP levels had been advanced as viable therapies.

Specifically, in reference to treatment of ALS, Ellis *et al.* (*J. Neurosci.* (2003) 23:43-51; submitted herewith) state that "[i]n the CNS, NO modulates cerebral blood flow and synaptic transmission via the activation of soluble guanylate cyclase and increases in cGMP (Murad, 1998) . . . [s]everal lines of evidence have emerged that suggest that NO can be either neuroprotective (Lipton et al., 1993; Chiueh, 1999) or neurodestructive (Dawson et al., 1991, 1992; Samdani et al., 1997)." At least in view of such knowledge of an asserted link between elevation of cGMP levels and a neuroprotective effect, the skilled artisan would have had no reason to doubt the objective truth of the statements of the instant specification in reference to use of the cGMP-stimulating compounds of the instant invention to treat ALS.

Similarly, a link between phosphodiesterase (PDE) inhibition and treatment of MS was well established at the time of filing. For example, regarding treatment of Th1-mediated autoimmune diseases, including MS, Bielekova *et al.* (*J. Immunology* (2000) 164:1117-1124; submitted herewith) identified "data demonstrat[ing] a favorable drug profile of PDE4 and PDE4 combined with PDE3 inhibitors for the treatment of Th1-mediated autoimmune disorders." Thus, treatment of MS *via* inhibition of PDEs and resultant elevation of cyclic nucleotide levels was known in the art at the time of filing. Whereas PDE4 selectively hydrolyzes cAMP, inhibition of PDEs that selectively hydrolyze cGMP has also been described for treatment of MS. Specifically, Uthayathas *et al.* (*Pharmacol Rep.* (2007) 59:150-63;

submitted herewith) state that "Sildenafil selectively inhibits PDE5 and increases the level of cGMP leading to beneficial effects in targeting some organs (Fig. 1). Interestingly, several lines of recent evidence indicate that sildenafil may offer novel strategy in the therapeutic treatment of age-related memory impairment, pain, pulmonary hypertension and multiple sclerosis." At least in view of the preceding, the skilled artisan would have had no reason to doubt the objective truth of the statements of the instant specification in reference to use of the cGMP-stimulating compounds of the instant invention to treat MS.

However, in the interest of furthering prosecution, none of the claims as amended relate to prevention of disease, and claim 1 does not include ALS or MS, while claim 11 is limited to the treatment of ALS and MS. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection of claims 1-9 under 35 U.S.C. § 112, first paragraph.

Double Patenting

The Office Action rejects claim 10 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of U.S. Patent No. 6,803,365. Applicants submit that this rejection is rendered moot by cancellation of claim 10.

Rejection Under 35 U.S.C. § 102(b)

The Office Action rejects claims 1-10 under 35 U.S.C. § 102(b) as allegedly anticipated by Niewohner *et al.* (WO 01/47929; English language equivalent U.S. Patent No. 6,803,365). Specifically, the Office Action alleges that "Niewohner et al. teach the imidazo[1,3,5]triazinine of formula 1 with the limitations of claims 4, 5 and 6." Applicants respectfully traverse this rejection and submit that claim 1 as amended, and claims 5 and 6 dependent therefrom, are directed to "treatment of diseases or syndromes selected from the group consisting of ***nitrate-induced tolerance, a disease of the eye, central retinal arterial occlusion, posterior ciliary arterial occlusion, central retinal venous occlusion, optical neuropathy, macular degeneration, diabetes, premature labor, preeclampsia, alopecia, psoriasis, renal syndrome, cystic fibrosis, cancer, age-associated learning and memory disturbance, age-associated memory loss, craniocerebral trauma, post-traumatic craniocerebral trauma, concentration disturbance in a child suffering from learning and memory problems, dementia associated with Lewy bodies; dementia associated with degeneration of the frontal lobes including Pick's***

syndrome, Parkinson's disease, progressive nuclear palsy, dementia associated with corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jacob dementia, new variant Creutzfeld-Jacob dementia, HIV dementia, schizophrenia associated with dementia and schizophrenia associated with Korsakoff's psychosis [with a] cGMP-stimulating compound." Claim 11 concerns the treatment of ***multiple sclerosis*** and ***amyolateral sclerosis (ALS).***

For a prior art reference to anticipate in terms of 35 U.S.C. § 102 a claimed invention, the prior art must teach ***each and every element*** of the claimed invention. *Lewmar Marine v. Barient*, 827 F.2d 744, 3 USPQ2d 1766 (Fed. Cir. 1987). Applicants respectfully submit that Niewohner *et al.* does not teach or suggest a method or pharmaceutical for treating "diseases or syndromes selected from the group consisting of ***nitrate-induced tolerance, a disease of the eye, central retinal arterial occlusion, posterior ciliary arterial occlusion, central retinal venous occlusion, optical neuropathy, macular degeneration, diabetes, premature labor, preeclampsia, alopecia, psoriasis, renal syndrome, cystic fibrosis, cancer, age-associated learning and memory disturbance, age-associated memory loss, craniocerebral trauma, post-traumatic craniocerebral trauma, concentration disturbance in a child suffering from learning and memory problems, dementia associated with Lewy bodies; dementia associated with degeneration of the frontal lobes including Pick's syndrome, Parkinson's disease, progressive nuclear palsy, dementia associated with corticobasal degenerationHuntington's disease, thalamic degeneration, Creutzfeld-Jacob dementia, new variant Creutzfeld-Jacob dementia, HIV dementia, schizophrenia associated with dementia and schizophrenia associated with Korsakoff's psychosis***," or ***multiple sclerosis*** and ***amyolateral sclerosis (ALS)*** as is required by the instant claims. Thus, Niewohner *et al.* does not teach or suggest each and every limitation of the claimed invention. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1-10 under 35 U.S.C. § 102(b).

Rejection Under 35 U.S.C. § 103(a)

The Office Action rejects claims 1-10 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Niewohner *et al.* Specifically, the Office Action alleges that "[i]t would have been obvious to a person of ordinary skill in the art at the time of applicant's invention to use the compound of Niewohner to treat coronary heart disease since Niewohner teach that the

compound can be used to treat many different types of cardiovascular diseases, which would clearly encompass coronary heart disease, or in the very least it would have been obvious to administer the claimed compound to treat coronary heart disease, since it clearly is a well known type of cardiovascular disease." Applicants respectfully traverse this rejection.

Solely in the interest of expediting prosecution of the instant invention, claim 1, and claims 5-6 depending therefrom, are amended to require treatment of "diseases or syndromes selected from the group consisting of ***nitrate-induced tolerance, a disease of the eye, central retinal arterial occlusion, posterior ciliary arterial occlusion, central retinal venous occlusion, optical neuropathy, macular degeneration, diabetes, premature labor, preeclampsia, alopecia, psoriasis, renal syndrome, cystic fibrosis, cancer, age-associated learning and memory disturbance, age-associated memory loss, craniocerebral trauma, post-traumatic craniocerebral trauma, concentration disturbance in a child suffering from learning and memory problems, dementia associated with Lewy bodies; dementia associated with degeneration of the frontal lobes including Pick's syndrome, Parkinson's disease, progressive nuclear palsy, dementia associated with corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jacob dementia, new variant Creutzfeld-Jacob dementia, HIV dementia, schizophrenia associated with dementia and schizophrenia associated with Korsakoff's psychosis***." Applicants submit that nitrate-induced tolerance, a disease of the eye, central retinal arterial occlusion, posterior ciliary arterial occlusion, central retinal venous occlusion, optical neuropathy, macular degeneration, diabetes, premature labor, preeclampsia, alopecia, psoriasis, renal syndrome, cystic fibrosis, cancer, age-associated learning and memory disturbance, age-associated memory loss, craniocerebral trauma, post-traumatic craniocerebral trauma, concentration disturbance in a child suffering from learning and memory problems, dementia associated with Lewy bodies; dementia associated with degeneration of the frontal lobes including Pick's syndrome, Parkinson's disease, progressive nuclear palsy, dementia associated with corticobasal degeneration, Huntington's disease, thalamic degeneration, Creutzfeld-Jacob dementia, new variant Creutzfeld-Jacob dementia, HIV dementia, schizophrenia associated with dementia and schizophrenia associated with Korsakoff's psychosis ***are not cardiovascular, cerebrovascular or urogenital diseases***. Similarly, claim 11 concerns the treatment of ALS and MS, which also are not ***cardiovascular, cerebrovascular or urogenital diseases***.

Rather, the diseases and syndromes recited in the instant claims are those that the instant specification describes treating by improving the microcirculation of a tissue which contains a cGMP-metabolizing phosphodiesterase *via* administration of an effective amount of a cGMP-stimulating compound. Applicants respectfully submit that it would not have been obvious to one of ordinary skill in the art in view of Niewohner *et al.* to treat the non-cardiovascular, non-cerebrovascular, non-urogenital diseases recited in the instant claims. Accordingly, Applicants request reconsideration and withdrawal of the rejection of claims 1-10 under 35 U.S.C. § 103(a).

CONCLUSION

In view of the above amendments and arguments, Applicants respectfully submit that all claims are allowable as written, and accordingly request early and favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicants' attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney.

Dated: January 7, 2008

Respectfully submitted,

By _____

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Global Loss of Na,K-ATPase and Its Nitric Oxide-Mediated Regulation in a Transgenic Mouse Model of Amyotrophic Lateral Sclerosis

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Na,K-ATPase plays a critical role in energy metabolism and ion fluxes. Its loss was investigated in the G93A mouse model of amyotrophic lateral sclerosis (ALS) in which the mutation of Cu/Zn superoxide dismutase (SOD1) is thought to lead to aberrant oxidative damage. Observed losses in spinal cord Na,K-ATPase activity exceeded all expectations. All three catalytic subunit isoforms (α1, α2, α3) were reduced, and the global α subunit loss affected not just neurons, glia, and myelinated axon tracts but even ependymal and pial membranes. Decreases in Na,K-ATPase activity were greater than losses of protein, and there were losses of Na,K-ATPase α, but not β, subunits. Together, these observations are consistent with selective degradation of the α subunit after damage. Overexpression of normal SOD1 does not cause ALS-like symptoms, but it has other known pathological effects. In transgenic mice overexpressed normal human SOD1 had a smaller but still considerable effect on Na,K-ATPase. Furthermore, the nitric oxide-mediated regulatory pathway for Na,K-ATPase inhibition was undetectable in spinal cord tissue slices from mice overexpressing either mutant or normal human SOD1. Na,K-ATPase activity did not respond to nitric oxide donors, and the free radical-dependent step of the pathway could not be bypassed by the addition of the downstream protein kinase G activator, 8-Br-cGMP. The data demonstrate that Na,K-ATPase is vulnerable to aberrant SOD1 activity, making it a potential contributing factor in disease pathology. Moreover, the global cellular distribution of Na,K-ATPase loss indicates that SOD1 overexpression is far-reaching in its pathological effects.

Key words: Na,K-ATPase; SOD1; amyotrophic lateral sclerosis; neurodegeneration; spinal cord; nitric oxide

Introduction

Amyotrophic lateral sclerosis (ALS) is an age-dependent motor neuron disease. Certain familial ALS cases are inherited as an autosomal dominant trait with mutations in cytosolic Cu/Zn superoxide dismutase 1 (SOD1) (Rosen et al., 1993; Brown, 1995). SOD1 normally converts superoxide, a by-product of mitochondrial metabolism, to water and hydrogen peroxide. Simple loss of SOD1 activity has been ruled out as a cause of the disease. Instead, there is evidence for "gain of toxic function," such as increases in copper-, free radical-, or oxidative damage, which is not alleviated by increases or decreases in the level of normal SOD1 activity (Cleveland and Rothstein, 2001; Julien, 2001).

The Na,K-ATPase consumes 50% of the energy supply in the CNS (Ames, 2000). Its catalytic (α) subunit is sensitive to damage by free radicals and other oxidative stress (Kim and Akera, 1987; Xie et al., 1995; Mense et al., 1997), and the oxidized Na,K-ATPase α subunit can be degraded by calpain, proteosomal, and lysosomal pathways (Zolotarjova et al., 1994; Thevenod and Friedmann, 1999). It thus may be one of the links between alterations in free radical homeostasis and ALS pathology. In other circumstances the inhibition of Na,K-ATPase increases the sensitivity of neurons to glutamate excitotoxicity because of complementary effects on neurons (enhancing glutamate effects and Ca^{2+} accumulation) and astrocytes (reducing the driving force for Na^+-dependent glutamate clearance) (Lees et al., 1990; Brines and Robbins, 1992; Brines et al., 1995; Calabresi et al., 1995; Lees and Leong, 1996; Stelmashook et al., 1999). Furthermore, the free radical nitric oxide ($NO^{\cdot}$) normally regulates the Na,K-ATPase via the activation of soluble guanylate cyclase and cGMP (McKee et al., 1994), a pathway that is shared by glutamate and oxygen free radicals in the CNS. This pathway forms a convergence point for the action of several intercellular and intracellular molecular messengers that have been implicated in neuronal viability under stress (Dawson et al., 1991; Nathanson et al., 1995). Together, these suggest that either loss or excessive inhibition of Na,K-ATPase could contribute to motor neuron death via direct oxidative damage or via the enhancement of $NO^{\cdot}$ and other free radical effects.

The Na,K-ATPase has two required subunits, α and β. There are three α isoforms (α1, α2, and α3) and three β isoforms (β1, β2, and β3) in the CNS (Sweadner, 1989; Blanco and Mercer, 1998), which have different kinetic properties and are likely to be regulated differently (Blanco and Mercer, 1998; Crambert et al., 2000). Although there are many exceptions to the rule, neurons have predominantly $\alpha 3\beta 1$ and astrocytes have predominantly $\alpha 2\beta 2$, whereas both neurons and glia can express $\alpha 1$.

To test the hypothesis that Na,K-ATPase loss occurs in a mouse model of ALS and in SOD1 overexpression, we used transgenic mice that express either human SOD1 with the G93A mis-

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sense mutation or normal human SOD1 over and above normal mouse SOD1 (Gurney et al., 1994).

Materials and Methods

Reagents. Routine reagents, sodium nitroprusside (SNP), superoxide dismutase, and ouabain were purchased from Sigma (St. Louis, MO). ^{1}H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), diethylenetriamine NO (DETA-NO), 8-Br-cGMP, and okadaic acid were obtained from Sigma-RBI (Natick, MA). The cGMP enzyme immunoassay system was purchased from Amersham Biosciences (Piscataway, NJ). Cy3-conjugated goat-anti mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The Puregene DNA Isolation Kit was purchased from Gentra (Minneapolis, MN).

Transgenic SOD1 mice. The mice were obtained from Dr. Robert H. Brown Jr. (Massachusetts General Hospital, Charlestown, MA) or The Jackson Laboratory (Bar Harbor, ME) and were strains originally developed by Dr. Mark Gurney (Gurney et al., 1994). The G1H strain of G93A mice carries high copy numbers of human SOD1, with a missense mutation substituting glycine with alanine at codon 93, and develops ALS-like symptoms between 3 and 4 months of age. This strain is maintained as a hemizygote hybrid line, progeny of a cross between C57BL/6J and SJL mice. Transgenic males were crossed with nontransgenic B6SJL-F1 females. Animals were genotyped by purifying mouse tail DNA with a Puregene DNA Isolation Kit; PCR products were separated on a 2% agarose gel. The controls were transgenic normal human SOD1 overexpressors of the B5SJL strain and nontransgenic littermates.

Na,K-ATPase activity and cGMP measurements. Whole spinal cord tissue was dissected, and tissue slices (0.4 × 0.4 × 1 mm) were prepared on a Brinkmann chopper cooled to 4°C and suspended (25–30 mg/ml wet weight) in microdissection buffer containing (in mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.25 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, and 2 NaOH, pH-adjusted to 7.4 at 34°C.

SNP, DETA-NO, or 8-Br-cGMP, when used, was added to tubes that contained 1 ml aliquots of slice suspension. Tubes were incubated for 15 min at 34°C with rocking and then frozen at −80°C. In studies that used ODQ or okadaic acid, the inhibitor was added 3 min before the addition of the other drug. Tubes were thawed and centrifuged (1700 × *g* for 15 min at 4°C), and the supernatant was removed and assayed for cGMP by using an enzyme immunoassay according to the manufacturer's instructions. The tissue slice pellets were resuspended in resuspension buffer containing (in mM): 85 NaCl, 20 KCl, 4 $MgCl_2$, 0.2 EGTA, and 30 histidine, pH-adjusted to 7.2.

Two similar ATPase assays were used. For most experiments with tissue from 4-month-old animals the Na,K-ATPase activity was determined by using the pyruvate kinase/lactate dehydrogenase assay that couples the generation of ADP and oxidation of NADH as described previously (Ellis et al., 2000). Treated tissue slices were homogenized with a ground glass homogenizer. Na,K-ATPase activity was calculated from the difference between the slopes in the time course of absorption change at 340 nm in the absence and the presence of 3 mM ouabain. For most experiments with tissue from 2-month-old animals the activity was determined by the colorimetric ATPase assay: ATP was hydrolyzed, and the released P_i was measured by forming a complex with molybdate. The pelleted tissue slices were resuspended and refrozen for at least 20 min at −80°C in 1 ml of resuspension buffer. Tubes were thawed on ice water. For further permeabilization saponin (20 μg/ml) was added, and the slices were incubated for 10 min at 34°C. Aliquots of tissue slices (~10–15 μg; 7.5–10 μl) were added to 300 μl of ATPase buffer containing (in mM): 3 ATP, 140 NaCl, 20 KCl, 3 $MgCl_2$, and 30 histidine, pH 7.2, with or without 3 mM ouabain. In this and previous work we verified the equivalence of the two assays that were used (Ellis et al., 2000).

Immunoblots. Spinal cord tissue was dissected from transgenic mutant SOD1 mice, transgenic normal human SOD1 overexpressors, and nontransgenic littermate controls and then homogenized at 4°C in microdissection buffer. Protein concentrations were determined by the Lowry method, and samples (50 μg of protein) were separated by gel electrophoresis on Laemmli gels and transferred to nitrocellulose membrane electrophoretically. For detection of Na,K-ATPase subunits the isoform-specific monoclonal antibodies used were 6F (for α1; Developmental Studies Hybridoma Bank, Iowa City, IA), McB2 (for α2), and XVI-F9G10 (for α3; Affinity BioReagents, Neshanic Station, NJ), all of which bind in the first 60 residues of the respective α subunits; rabbit polyclonal antibodies SpETb1 and SpETb2 (gift of P. Martin-Vasallo, University of Tenerife, Spain) were used for β1 and β2, respectively. Anti-KETYY against the α C terminus (gift of Dr. J. Kyte, University of California, San Diego, CA) was used to detect all α isoforms together. Anti-superoxide dismutase (Cu/Zn) polyclonal antibody specific to human SOD1 (Calbiochem-Novabiochem, San Diego, CA) was used to quantify the level of overexpressed enzyme. Blots subsequently were stained with horseradish peroxidase-conjugated secondary antibody, developed with luminol reagent, and quantified with a Molecular Dynamics scanning densitometer (Sunnyvale, CA).

Immunofluorescence. Nontransgenic controls and transgenic mutant SOD1 mice were anesthetized and perfused with PBS, followed by periodate-lysine-paraformaldehyde fixative for 20 min (McLean and Nakane, 1974). The spinal cords encased in the vertebral columns were harvested and postfixed for 48 hr. The spinal cords were removed and washed in PBS overnight and then soaked in 25% sucrose in 0.1 M PBS for 24 hr before sectioning. Cryostat sections (10–14 μm) were cut at −20°C and collected on positively charged slides (ProbeOn Plus, Fisher Scientific, Durham, NC). Slides were washed in 0.1 M PBS and incubated in blocking buffer containing 0.1% Triton X-100 and 5% normal goat serum in PBS. Tissue sections were incubated with primary monoclonal antibodies for the α subunits described above, washed, and stained with secondary antibody Cy3-conjugated goat-anti mouse IgG. For β1, monoclonal antibody BSP-3 was used, a gift of Dr. C. Goridis (INSERM-CNRS, Marseille Luminy, France). For β2, monoclonal antibody 426 was used, a gift of Dr. Melitta Schachner (University of Hamburg, Germany). The images were viewed with a Zeiss confocal microscope (Oberkochen, Germany).

Statistics. Statistical comparisons were performed by ANOVA, followed by Fisher's protected least significant difference (PLSD) and Scheffé's *F* test for comparison of significant difference among different means.

Results

Decreased ouabain-sensitive Na,K-ATPase activity in transgenic mutant SOD1 mice

Ouabain-sensitive Na,K-ATPase activity was measured in spinal cord tissue slice homogenates of transgenic mice expressing mutant or normal human SOD1 and wild-type controls. Because the assay is performed *in vitro* with saturating levels of ATP and ions, the measured activity reflects the maximal velocity of the enzyme itself and not ATP depletion secondary to mitochondrial damage *in vivo*. Figure 1*A* shows that Na,K-ATPase activity was decreased remarkably (70–75%) in the severely impaired 4-month-old mutant SOD1 mice compared with nontransgenic animals. To determine whether the decreases were a result of the mutation or of overexpression of SOD1, we measured activity in samples from transgenic mice overexpressing normal human SOD1. There was a large but less dramatic decrease (40–45%) in ouabain-sensitive Na,K-ATPase activity in the overexpressors. Immunoblot and densitometric analysis with a species-specific antibody for human SOD1 showed that the total level of exogenous SOD1, compared with a sample of purified enzyme, was not statistically different in mice overexpressing mutant and normal human SOD1 (Fig. 1*B,C*). The greater Na,K-ATPase activity loss in the mutant is consistent with "gain of function" consequences of excess SOD1 that are exacerbated by the mutation.

Cerebellum is not implicated in neurodegenerative processes in this ALS mouse model (Almer et al., 1999), but comparable losses in Na,K-ATPase activity also were seen in cerebellum in

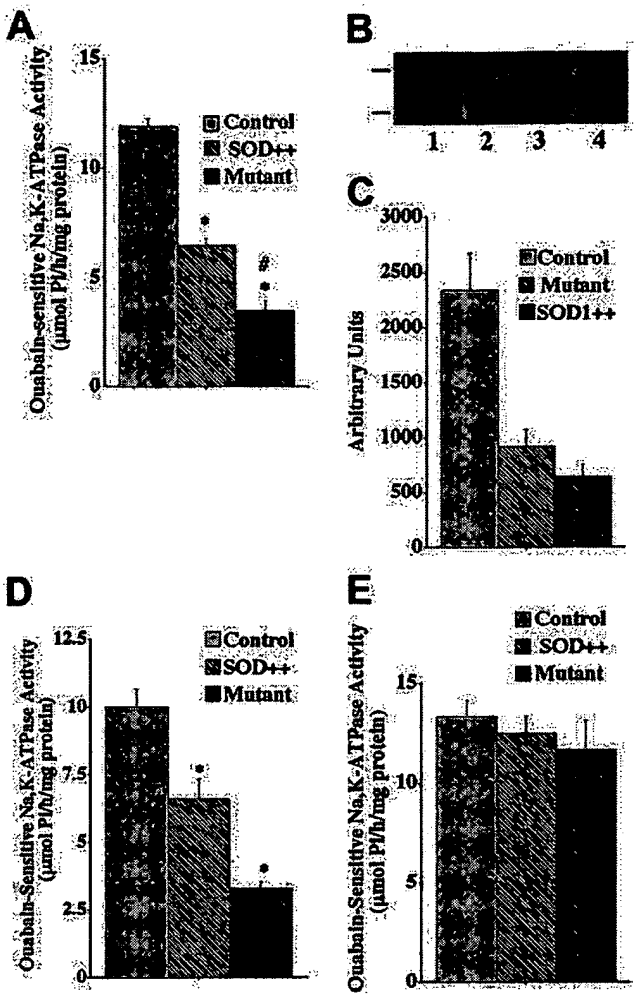


Figure 1. Ouabain-sensitive Na,K-ATPase activity in spinal cord and cerebellum of nontransgenic controls (*Control*), transgenic normal human SOD1 overexpressors (*SOD++*), and transgenic SOD1 mice (*Mutant*), and expression of normal and mutant human SOD1. For *A* and *D*, activity is expressed as μmol P_i/hr per milligram of protein. *A*, Spinal cord tissue slices from 4-month-old animals were homogenized, and ouabain-sensitive Na,K-dependent hydrolysis of ATP was determined. Values for activity represent the means ± SEM for an average of three samples in five experiments. *Significantly different from control at $p < 0.05$ (by ANOVA, Fisher's PLSD, and Scheffé's *F* test). #Significantly different from transgenic normal human SOD1 overexpressors at $p < 0.05$ (by ANOVA and Fisher's PLSD). *B*, Immunoblot detection of human SOD1 in nontransgenic controls (*lane 2*), transgenic mutant SOD1 mice (*lane 3*), normal human SOD1 overexpressors (*lane 4*), and purified human SOD1 as a positive gel control (*lane 1*). *C*, Densitometric analysis of human SOD1 expression levels in transgenic mutant SOD1 mice (*Mutant*), transgenic normal human SOD1 overexpressors (*SOD++*), and purified human SOD1 as a positive gel control. Values are expressed as arbitrary units and represent the means ± SEM for an average of three experiments. *D*, Na,K-ATPase activity was measured from cerebellar tissue slices from 4-month-old animals. Values represent the means ± SEM for an average of three samples in six experiments. *Significantly different from control at $p < 0.05$ (by ANOVA, Fisher's PLSD, and Scheffé's *F* test). *E*, Na,K-ATPase activity was measured in spinal cord tissue slice preparations from 2-month-old animals. Values for ouabain-sensitive Na,K-ATPase activity represent the means ± SEM for an average of three samples in three experiments.

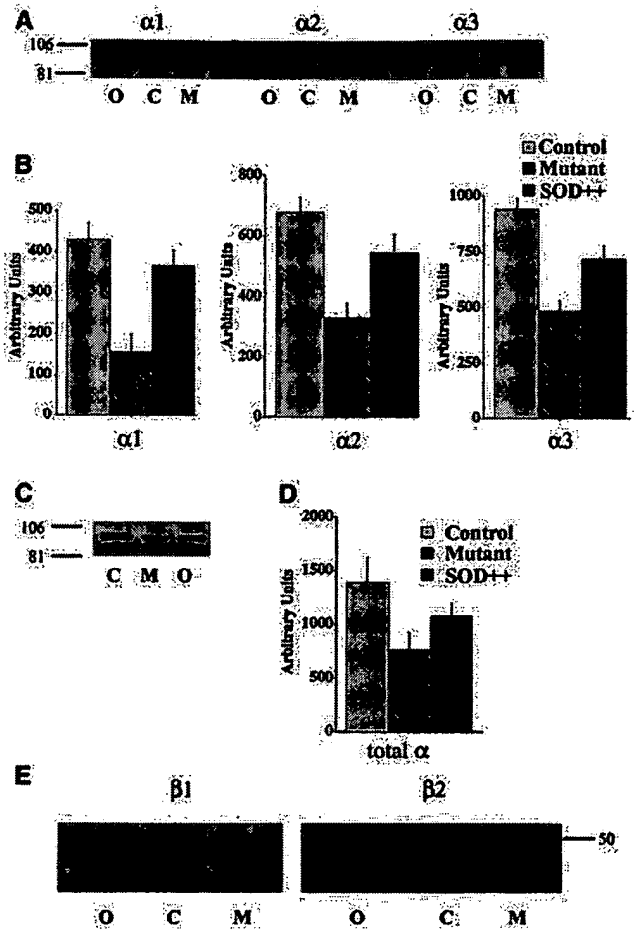
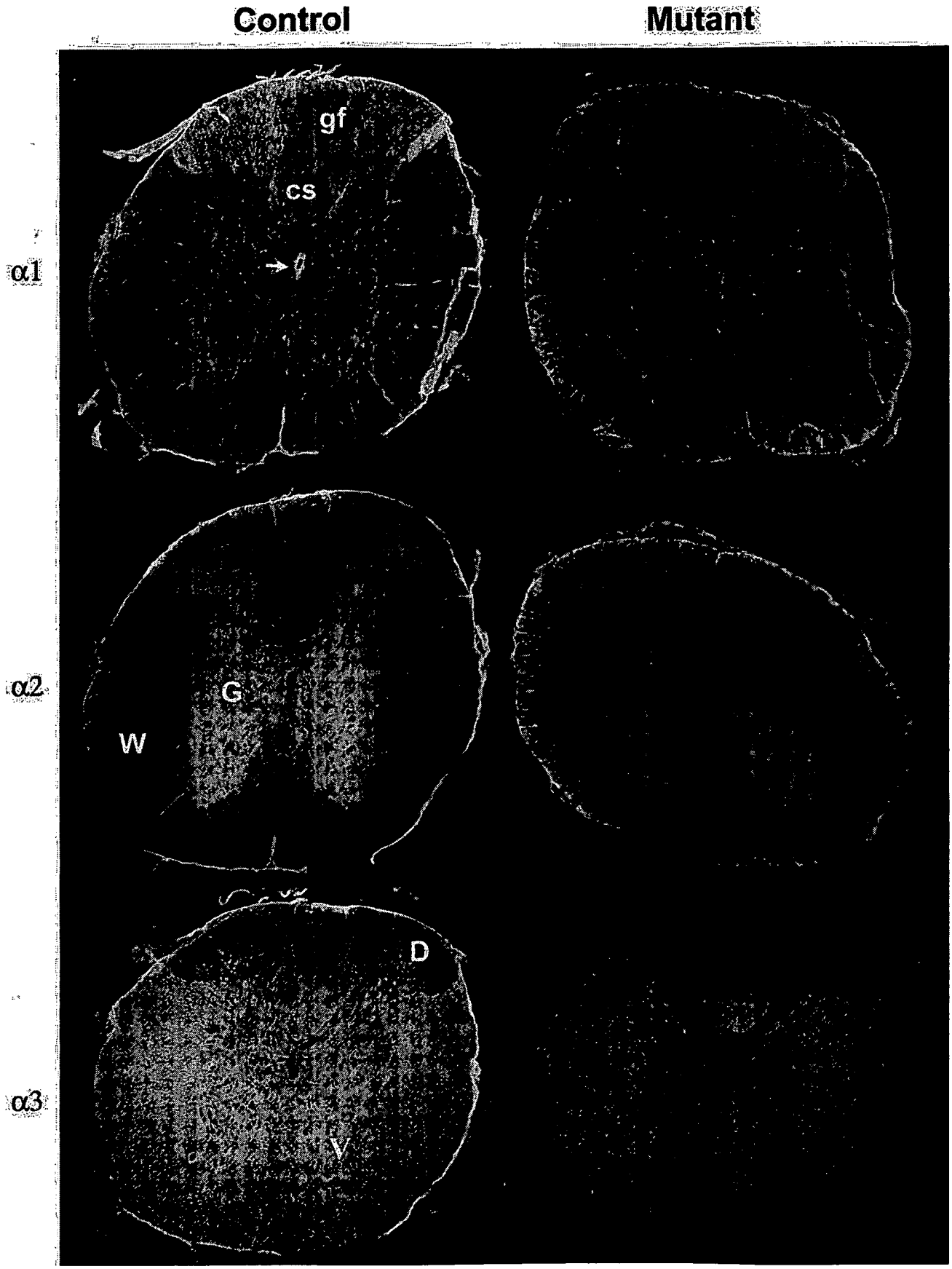


Figure 2. Immunoblot detection and quantitative analysis of Na,K-ATPase isoforms in nontransgenic controls (*C*, control), transgenic mutant SOD1 mice (*M*, mutant) and transgenic normal human SOD1 overexpressors (*O*, SOD++). *A*, *B*, Immunoblot and densitometric analysis of α1, α2, α3. For all immunoblots 50 μg of spinal cord homogenates was used. *A*, Separated protein was stained with monoclonal antibodies to α1 (6F), α2 (McB2), and α3 (XVIF9G10). Crude homogenates from rat brain served as positive controls for the antibodies (data not shown). Molecular weight markers are shown in kDa. *B*, Data are expressed as arbitrary units and represent the means ± SEM for an average of three experiments. *C*, Immunoblot stained with KETYY, an antibody that recognizes all Na,K-ATPase α subunits. *D*, Densitometric analysis of KETYY expressed in arbitrary units and representing the means ± SEM for an average of three experiments. *E*, Spinal cord homogenates were stained for β1 (SpETb1) and β2 (SpETb2) polyclonal antibodies. The data that are shown are representative of multiple experiments.

4-month-old animals (Fig. 1*D*). In agreement with Almer et al. (1999), we did not see any indication of major neuron loss in cerebellar tissue sections (data not shown). This is an initial indication that the losses of activity were not attributable simply to a loss of neurons. Figure 1*E* shows that there were no significant reductions in Na,K-ATPase activity in spinal cord of transgenic mutant mice or transgenic overexpressors at 2 months of age, before the onset of obvious neurological symptoms.

Decreased Na,K-ATPase α subunits in transgenic mutant SOD1 mice

Losses in Na,K-ATPase activity could be attributable to enzyme inactivation, protein degradation, changes in gene expression, failure to transport newly synthesized protein to the axon, loss of neurons, or a combination of these. Quantitative detection of Na,K-ATPase subunits makes it possible to assess whether protein levels were changed as much as activity. It also allows some conclusions to be made about whether the effect is on a particular Na,K-ATPase isoform or a particular cell type, because the α3 subunit is all in neurons and the myelinated axon tracts, whereas most of α2 is in astrocytes (Hieber et al., 1991; McGrail et al., 1991; Watts et al., 1991; Peng et al., 1997). The relative content of Na,K-ATPase α1, α2, and α3 subunits was determined in ho-


Control
Mutant
gf
cs
α1
G
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α3

mogenates of spinal cord of transgenic mutant SOD1 mice, transgenic normal human SOD1 overexpressors, and nontransgenic controls by using isoform-specific monoclonal antibodies. Immunoblots (Fig. 2*A*) and densitometric analysis (Fig. 2*B*) showed that there were decreases in α1 (65%), α2 (50%), and α3 (50%) in transgenic mutant SOD1 mice compared with nontransgenic controls. Although there were also decreases in the three α isoforms in transgenic normal human SOD1 overexpressors, the decreases were smaller compared with transgenic mutant mice (Fig. 2*B*). Total Na,K-ATPase α subunit level was quantified with a polyclonal antibody, anti-KETYY, that recognizes all Na,K-ATPase isoforms. Figure 2, *C* and *D*, shows that there were close to 50% decreases in total α protein in transgenic mutant SOD1 mice when compared with nontransgenic controls and 20–25% decreases in normal SOD1 overexpressors. Isoform-specific antibodies for the β1 and β2 Na,K-ATPase subunits were used to examine β levels. In contrast to the α subunit, no differences were detected in β1 (mostly in neurons) or β2 (mostly in astrocytes) in spinal cord samples from transgenic mutant SOD1, transgenic normal human SOD1 overexpressors, or nontransgenic control animals (Fig. 2*E*). β3, which is found only in oligodendrocytes and at a low level (Martin-Vasallo et al., 2000), was not examined.

The reduction in Na,K-ATPase activity (Fig. 1*A*) exceeded the reduction in total Na,K-ATPase α subunit (Fig. 2*D*) in both mutant SOD1 mice and normal human SOD1 overexpressors by a substantial amount. Both measures were expressed per milligram of protein, and so the losses were over and above any generalized loss of tissue mass, which does occur with the loss of motor neurons. The greater loss of ATP hydrolysis than α subunit indicates that perturbations of SOD1 activity have an acute effect on Na,K-ATPase apart from any effect on gene expression, tissue or axon atrophy, or cell loss.

Na,K-ATPase isoform distribution in normal and mutant spinal cord

The distribution of the various Na,K-ATPase α subunits was determined by using confocal immunofluorescence with isoform-specific monoclonal antibodies. In the dorsal horn of the rat spinal cord some large neurons express both α2 and α3, whereas in the ventral horn some motor neurons express α1 and α3 and the rest just α3 (Mata et al., 1991; McGrail et al., 1991; Watts et al., 1991). In the mice that were investigated here, the pattern of staining for each α isoform was identical in sections from all levels of the spinal cord (cervical, thoracic, lumbar, and sacral; data not shown) except for the underlying structural differences in dorsal and ventral horns and axon tracts at different levels. Figure 3 shows immunostaining of tissue sections from the lumbar expansion of the spinal cord in 4-month-old nontransgenic controls and transgenic mutant SOD1 mice. In keeping with the plasma membrane location of the Na,K-ATPase, the cytoplasm of large-diameter neuronal cell bodies was unstained, although there were α3 ring-stained cell bodies in the central and lower regions of the dorsal horn. α2 stain characteristic of astrocytes was brightest in the gray matter, but it extended outward into the myelinated tracts accompanying radiating bundles of axons. It was notable that there were differences in the distribution of Na,K-ATPase isoforms in wild-type mouse spinal cord that have not been described before. The most striking was an apparent complementary difference in predominant α isoforms between dorsal and ventral horn. The parenchyma of dorsal horn stained most brightly for α1, whereas ventral horn stained most brightly for α3. The complementary difference also was observed for axons in the dorsal columns. The dorsal one-half of the dorsal column, the gracile fascicle that consists of ascending sensory axons, was enriched in α1, and the ventral one-half of the dorsal column, a descending corticospinal tract, was enriched in α3 as well as α1. These and the lateral α3-containing axon tracts are presumably the origin of the unidentified α1- versus α3-containing myelinated tracts observed previously in the rat medulla (McGrail et al., 1991). In addition, the endothelial cells that line the central canal were stained brightly for α1, but not for α2 or α3, whereas the ensheathing pial membranes and fragments of dura mater stained for all three isoforms.

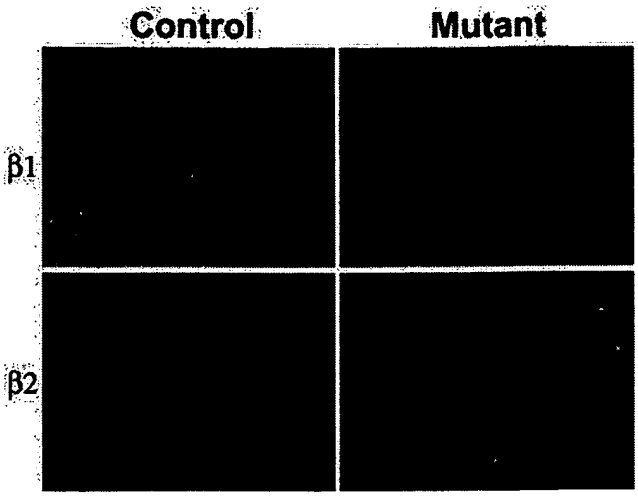

Figure 4. Immunodetection of Na,K-ATPase β isoforms in nontransgenic controls and transgenic mutant SOD1 mice. Tissue sections were labeled with monoclonal antibodies to β1 (BSP-3) and β2 (426).

When they were compared with nontransgenic controls, there were markedly lower levels of staining for all three Na,K-ATPase α isoforms in transgenic mutant SOD1 animals (Fig. 3). If losses in Na,K-ATPase had been attributable to specific cell loss or to the atrophy of axons, the remaining cells would have more normal stain intensity. The uniformity of the loss of stain was the most notable feature, extending even to the ependymal lining of the central canal. The pial membranes, which appeared somewhat disrupted in the mutant mice, penetrated the white matter tracts more than they should, and their stain for α3 virtually was abolished.

In contrast, and consistent with the immunoblots shown above, staining patterns for β1 and β2 subunits were unaltered in the transgenic mutant SOD1 mice (Fig. 4). The principal difference that was seen was in the diffusely shrunken appearance of the pathological spinal cord. A lack of effect on β expression suggests a lack of effect on Na,K-ATPase expression and biosynthesis, as discussed below.

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Figure 3. Immunofluorescence detection of Na,K-ATPase isoforms in nontransgenic controls and transgenic mutant SOD1 mice. Tissue sections were labeled with monoclonal antibodies to α1 (6F), α2 (McB2), and α3 (XVIF9G10). Images were taken at 10×, and montages were made to display the entire section. *D*, Dorsal horn; *G*, gray matter; *W*, white matter; *V*, ventral horn; *gf*, gracile fascicle of ascending sensory axons; *cs*, corticospinal tract. The *arrow* points to the ependyma lining the central canal.

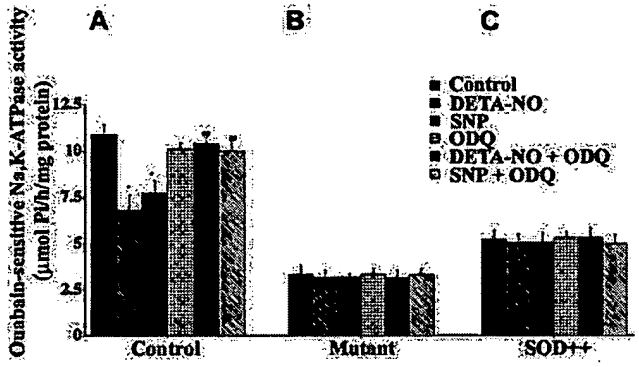

Figure 5. SNP- and DETA-NO-induced inhibition of ouabain-sensitive Na,K-ATPase was abolished in transgenic mutant SOD1 mice and transgenic normal human SOD1 overexpressors. Spinal cord tissue slices were incubated with or without ODQ (1 μM) for 3 min at 34°C, followed by incubation with DETA-NO (100 μM) or SNP (100 μM) for 15 min at 34°C. Drugs were removed, tissue slices were homogenized, and ouabain-sensitive Na,K-ATPase activity was measured. For all graphs that are shown, activity is expressed as μmol P_i/hr per milligram of protein, and values represent the means ± SEM for experiments on three animals done in triplicate. *A*, Ouabain-sensitive Na,K-ATPase activity in nontransgenic controls. *Significantly different from the control at $p < 0.05$ (by ANOVA and Fisher's PLSD). ##Significantly different from DETA-NO- and SNP-treated samples at $p < 0.05$ (by ANOVA and Fisher's PLSD). *B*, *C*, Ouabain-sensitive Na,K-ATPase activity in transgenic mutant SOD1 and transgenic SOD1 normal human SOD1 overexpressors.

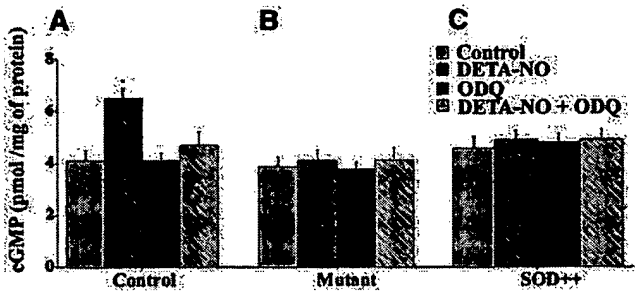

Figure 6. cGMP levels are unaltered in transgenic SOD1 mice in response to DETA-NO treatment. Spinal cord tissue slices from nontransgenic controls (*A*), transgenic mutant SOD1 mice (*B*), or transgenic normal human SOD1 overexpressors (*C*) were incubated for 3 min at 34°C with ODQ (1 μM), followed by incubation for 15 min at 34°C with DETA-NO (100 μM). After centrifugation the supernatant was removed and assayed for cGMP, expressed as pmol/mg protein. *Significantly different from the control group at $p < 0.05$ (by ANOVA, Fisher's PLSD, and Scheffé's *F* test). Values for cGMP levels represent the means ± SEM for three experiments.

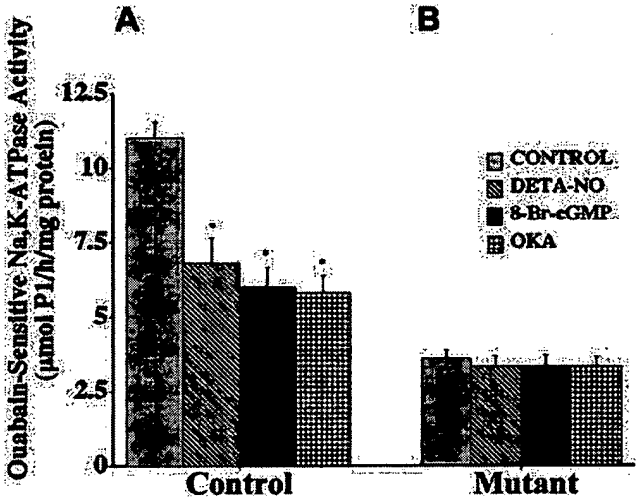

Figure 7. Ouabain-sensitive Na^+,K-ATPase activity in spinal cord of nontransgenic control mice (*A*) and transgenic mutant SOD1 mice (*B*) after incubation with DETA-NO (100 μM), 8-Br-cGMP (2 mM), or okadaic acid (*OKA*; 400 nM). Results are expressed as μmol P_i/hr per milligram of protein, and values represent the means ± SEM for three animals done in triplicate. *Significantly different from the control at $p < 0.05$ (by ANOVA and Fisher's PLSD).

NO is unable to regulate Na,K-ATPase activity in spinal cord of transgenic mice

In some tissues NO is a normal Na,K-ATPase regulator acting through cGMP. Because free radical homeostasis appears to be perturbed in some forms of ALS, we tested whether regulation of the Na,K-ATPase was affected in this model. Spinal cord tissue slices from nontransgenic control mice were exposed to the NO donors SNP (Garthwaite et al., 1995) and DETA-NO (Diodati et al., 1993) for 15 min. Both donors caused a marked reduction (35–45%) of ouabain-sensitive Na,K-ATPase activity (Fig. 5*A*). The effects were specific to Na,K-ATPase, because no measurable changes were observed in the ouabain-insensitive (Mg-ATPase) activity (data not shown). Many of the physiological actions of NO on Na,K-ATPase activity involve activation of soluble guanylate cyclase (McKee et al., 1994; Nathanson et al., 1995; Scavone et al., 1995; Ellis et al., 2000, 2001). When SNP- or DETA-NO-treated spinal cord tissue slices were exposed to ODQ, an inhibitor selective for soluble guanylate cyclase (Garthwaite et al., 1995), it primarily blocked the SNP- and DETA-NO-induced inhibition of ouabain-sensitive Na,K-ATPase activity (Fig. 5*A*).

The effects of NO donors on activity in tissue slices from spinal cord of transgenic mutant SOD1 mice and of transgenic normal human SOD1 overexpressors are shown in Figure 5, *B* and *C*. The basal Na,K-ATPase activity was low, and neither SNP nor DETA-NO further inhibited it. The addition of ODQ in the presence of SNP or DETA-NO, or alone, failed to alter ouabain-sensitive Na,K-ATPase activity in spinal cord tissue slices of transgenic mutant SOD1 mice (Fig. 5*B*) or transgenic normal human SOD1 overexpressors (Fig. 5*C*). Because ODQ treatment did not restore the lost activity, the data argue against a high basal level of otherwise-normal NO-mediated regulation as the cause of Na,K-ATPase inhibition in the transgenic mice. These results indicate a surprisingly complete perturbation, in normal SOD1 overexpressors as well as mutants, of a regulatory pathway that in physiological conditions depends on the diffusion of NO from its site of synthesis to soluble guanylate cyclase.

The ability of NO donors to increase levels of cGMP by activating soluble guanylate cyclase was tested in the three groups of mice. In nontransgenic control mice the addition of DETA-NO to spinal cord tissue slices caused a 40% increase in cGMP levels that, as expected, was abolished by the guanylate cyclase inhibitor ODQ (Fig. 6*A*). In contrast, there were no measurable changes in cGMP levels in either transgenic mutant SOD1 mice or transgenic normal human SOD1 overexpressors treated with DETA-NO, ODQ, or DETA-NO plus ODQ (Fig. 6*B*,*C*). This could mean that soluble guanylate cyclase, like Na,K-ATPase, is inactivated in these mice, possibly targeted by its specific binding site for a free radical, although such losses were not reported in the G1L strain of G93A mice, which expresses mutant SOD1 at a lower level (Facchinetti et al., 1999). Alternatively, the NO generated by the artificial donors could be consumed rapidly in reactions catalyzed by the aberrant elevated levels of SOD1.

The pathway downstream of guanylate cyclase was tested by exposure of spinal cord tissue slices to the permeable protein kinase G activator, 8-Br-cGMP. Figure 7*A* shows that in nontransgenic controls this caused an inhibition of Na,K-ATPase activity as effective as DETA-NO treatment. However, there were

no changes in activity in tissue from transgenic mutant SOD1 mice (Fig. 7*B*). Evidence for the involvement of protein phosphorylation in mediating the normal NO-induced regulation of Na,K-ATPase activity is shown in Figure 7 also. The addition of okadaic acid (400 nM) at concentrations known to inhibit protein phosphatases type 1 and type 2A mimicked the effects of DETA-NO and 8-Br-cGMP in inhibiting ouabain-sensitive Na,K-ATPase activity in nontransgenic SOD1 controls (Fig. 7*A*). Okadaic acid had no effect, however, on transgenic mutant SOD1 mice (Fig. 7*B*). The inability to bypass any step of the NO-mediated regulatory pathway is evidence either for the broadly compromised state of the transgenic spinal cord or for the resistance of the residual Na,K-ATPase activity to any further regulation.

Discussion

The mechanisms of motor neuron death in ALS are unknown, but several theories have been proposed, including that mutations in SOD1 may result in oxidative stress (Beckman et al., 1993; Crow et al., 1997; Andrus et al., 1998), increases in NO synthase and reactivity of astrocytes (Almer et al., 1999), and glutamate excitotoxicity secondary to glutamate transporter defects (Rothstein et al., 1993; Bruijn et al., 1997; Trotti et al., 1999). Other possible causes of neuronal degeneration include alterations in mitochondrial function (Beal, 1995; Klivenyi et al., 1999), formation of mutant SOD1 protein aggregates (Bruijn et al., 1998), and improper assembly of intermediate filaments that have particular impact on motor neurons because of their exceptionally long axons (for review, see Cleveland and Rothstein, 2001; Julien, 2001). Although all of these may contribute to the pathology and some may determine the selective vulnerability of motor neurons, perturbation of free radical homeostasis directly related to the SOD1 mutation is the phenomenon that is least likely to be secondary to the neurodegeneration process. Curiously, selective expression of mutant SOD1 in either astrocytes or neurons alone has failed to induce the disease (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002).

Uniform loss of Na,K-ATPase

These studies demonstrate that there are surprisingly large decreases in ouabain-sensitive Na,K-ATPase in spinal cord of transgenic mutant SOD1 mice, as assessed by enzyme activity, α subunit content, and anatomical distribution. Because of the uniformity, the losses are not likely to be a secondary consequence of neuron atrophy or death; this was confirmed by the observation of similar losses of Na,K-ATPase in the cerebellum. There were less severe decreases in Na,K-ATPase in the mice overexpressing transgenic normal human SOD1. SOD1 overexpression, which occurs in Down's syndrome as a direct result of trisomy, also is thought to increase oxidative damage (Ceballos-Picot et al., 1991; Lee et al., 2001), exacerbate excitotoxicity (Bar-Peled et al., 1996), and affect hippocampal ultrastructure and function (Barkats et al., 1993; Gahtan et al., 1998). Because the transgenic normal human SOD1 mice we used do not show hallmarks of ALS pathology such as SOD1 aggregates, intermediate filament aggregates, and mitochondrial defects, the observed Na,K-ATPase losses are more likely to be attributable to a "gain of function" abnormality of free radical or oxidant homeostasis than to ALS-associated neurodegeneration. These findings suggest that decreased Na,K-ATPase activity contributes to SOD1 ALS via inactivation and the loss of all three α subunits.

Any given cell type expresses a particular combination of α and β subunits (McGrail et al., 1991; Watts et al., 1991; Peng et al., 1997; Wetzel et al., 1999; Martin-Vasallo et al., 2000). Many neurons, for example, express $\alpha3\beta1$, mature astrocytes express $\alpha2\beta2$, and oligodendrocytes express $\alpha2\beta3$, but there are exceptions, such as the granule neurons of the cerebellum that express $\alpha3\beta2$, Müller glial cells that express $\alpha1\beta2$, and neurons that express more than one assembled complex. Decreases in $\alpha3$ are diagnostic for neuronal defects, because this isoform is not expressed in glia. Decreases in $\alpha2$ are additional evidence that astrocytes as well as neurons are affected in the disease. Two well established functions of glia are the clearance of potassium and the Na^+-dependent uptake of glutamate from the extracellular space after synaptic activation. Na,K-ATPase is activated when $[Na^+]_i$ rises concomitantly with glutamate uptake (Rose and Ransom, 1996), and this stimulates the uptake of K^+. The importance of these observations to ALS is highlighted by reports of selective loss of the astrocyte-specific glutamate transporter EAAT2 (GLT-1) (Rothstein et al., 1995) and of its vulnerability to oxidation by certain mutant SOD1 forms (Trotti et al., 1999). A defect in the glutamate transporter, a defect in the underlying glial sodium pump activity, and a defect in Na,K-ATPase activity in the neurons themselves are compatible with previous proposals that excitotoxicity contributes to the progression of ALS.

Detection of the simultaneous loss of Na,K-ATPase α isoforms specific to all different cell types in the spinal cord paints a compelling picture of a defect that is not confined to the motor neurons that die, however. The implications of the diffuse nature of the Na,K-ATPase alterations are potentially far-reaching. It suggests, for one thing, that the mutant mice have a "sick cord" and that studies of almost any candidate protein could reveal alterations that may contribute to the final pathology. It also suggests that alterations in the barrier organs such as the ependyma and pia may contribute to the disease. The loss of one-half to three-quarters of the enzyme that is the largest consumer of ATP may help to offset the effects of mitochondrial pathology and suggests that further investigation of energy metabolism in ALS would be fruitful (Browne et al., 1998; Klivenyi et al., 1999; Ames, 2000).

Altered free radical homeostasis and regulation of Na,K-ATPase

The NO/soluble guanylate cyclase pathway was altered severely in both transgenic mutant human SOD1 mice and transgenic normal human SOD1 overexpressors, whereas it inhibited ouabain-sensitive Na,K-ATPase activity in nontransgenic control mice. NO-mediated regulation of Na,K-ATPase activity is known in other tissues, including ciliary process and choroid plexus (Ellis et al., 2000, 2001). In the CNS, NO modulates cerebral blood flow and synaptic transmission via the activation of soluble guanylate cyclase and increases in cGMP (Murad, 1998). The loss of NO regulation in transgenic mutant SOD1 mice might have been predicted, considering the severity of the illness. However, the total blockade of the NO/cGMP pathway for Na,K-ATPase regulation in transgenic normal SOD1 overexpressors was unexpected.

The role of NO in CNS-related diseases is not completely clear. Several lines of evidence have emerged that suggest that NO can be either neuroprotective (Lipton et al., 1993; Chiueh, 1999) or neurodestructive (Dawson et al., 1991, 1992; Samdani et al., 1997). NO generated from NO donors or synthesized endogenously after activation of the ionotropic glutamate receptor (Lafon-Cazal et al., 1993) can lead to neurotoxicity in part by reaction with superoxide anion and the subsequent formation of peroxynitrite (Lipton et al., 1993; Beckman and Koppenol, 1996).

In contrast, other studies have demonstrated that the neuroprotective effects of NO in CNS may result from nitration or nitrosylation of iron- or thiol-containing proteins (Stamler et al., 1992; Lipton et al., 1993; Chiueh, 1999). Such chemical modifications may alter significantly the biological activity of the protein and minimize the generation of reactive oxygen species and associated oxidative stress. A lack of effect on ALS pathology when G93A mice were crossed with neuronal nitric oxide synthase (nNOS) knock-out mice suggested no involvement, but residual nNOS activity because of the synthesis of truncated forms complicated the interpretation (Facchinetti et al., 1999).

The degree of inhibition of Na,K-ATPase activity in transgenic mutant mice exceeded the decrease in the α subunit. The Na,K-ATPase can be inhibited chemically by oxygen free radicals and their by-products (Mense et al., 1997). For example, superoxide anion, hydrogen peroxide, and hydroxyl radicals inhibited Na,K-ATPase activity, and this decrease correlated with increased lipid peroxidation (Viani et al., 1991; Huang et al., 1992). Of interest is the finding that various NO donors (excluding sodium nitroprusside) caused substantial direct alterations in Na,K-ATPase activity via reaction with free sulfhydryl groups (Boldyrev et al., 1997; Sato et al., 1997). Furthermore, as with other proteins (Stadtman, 1992), exposure of the Na,K-ATPase to free radicals made it more susceptible to degradation by proteolytic enzymes (Huang et al., 1992; Thevenod and Friedmann, 1999). Because α and β subunits normally are found in a 1:1 ratio, the reduction in α, but not β, was unexpected. The data are consistent with greater vulnerability of the α subunit to damage and degradation. This is plausible, considering that the majority of the mass of the β subunit is in the extracellular space where it is adapted to a more oxidizing environment than the cytoplasm, and that its six extracellular sulfhydryl groups are all in buried disulfide bonds. Most of the mass of the α subunit, in contrast, is in the reducing environment on the cytoplasmic side of the membrane, and it has 23 free sulfhydryls and other oxidizable groups.

The widespread defects in Na,K-ATPase, not confined to either neurons or glia, support the original hypothesis that perturbation of free radical homeostasis is the most likely root cause of mutant SOD1 ALS pathology. The abrogation of the NO/cGMP pathway of Na,K-ATPase regulation is an unexpected and potentially important event, whether it is a parallel defect with the same root cause or a causative step in Na,K-ATPase loss.

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Therapeutic Potential of Phosphodiesterase-4 and -3 Inhibitors in Th1-Mediated Autoimmune Diseases

Bibiana Bielekova, Anne Lincoln, Henry McFarland, and Roland Martin[1]

Phosphodiesterase-4 (PDE4) inhibitors have the potential to modulate immune responses from the Th1 toward the Th2 phenotype and are considered candidate therapies for Th1-mediated autoimmune disorders. However, depending on the model and cell types employed, studies of atopic individuals have come to the opposite conclusion, i.e., that PDE inhibitors may be beneficial in asthma. Using in vitro immunopharmacologic techniques we analyzed the effects of PDE4 and PDE3 inhibitors on human immune cells to address these discrepancies and broaden our understanding of their mechanism of action. Our results indicate that PDE inhibitors have complex inhibitory effects within in vivo achievable concentration ranges on Th1-mediated immunity, whereas Th2-mediated responses are mostly unaffected or enhanced. The Th2 skewing of the developing immune response is explained by the effects of PDE inhibitors on several factors contributing to T cell priming: the cytokine milieu; the type of costimulatory signal, i.e., up-regulation of CD86 and down-regulation of CD80; and the Ag avidity. The combination of PDE4 and PDE3 inhibitors expresses synergistic effects and may broaden the therapeutic window. Finally, we observed a differential sensitivity to PDE inhibition in autoreactive vs foreign Ag-specific T cells and cells derived from multiple sclerosis patients vs those derived from healthy donors. This suggests that PDE inhibition weakens the strength of the T cell stimulus and corrects the underlying disease-associated cytokine skew in T cell-mediated autoimmune disorders. These new findings broaden the understanding of the immunomodulatory actions of PDE inhibitors and underscore their promising drug profile for the treatment of autoimmune disorders. ***The Journal of Immunology*****, 2000, 164: 1117–1124.**

Hyperactive Th1-mediated immune responses are thought to be involved in the pathogenesis of many autoimmune diseases, including multiple sclerosis (MS).[2] Phosphodiesterases (PDE) are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes (1). There are 10 different PDE families, but immune cells predominantly express families PDE4, PDE3, and, to a lesser extent, PDE7 (2, 3). Although no PDE7 inhibitor is available, the inhibitors of PDE4 and PDE3 families exert complex immunomodulatory properties. In animals, these drugs inhibit Ag-mediated T cell proliferation and skew the T cell cytokine profile toward a Th2 phenotype by down-regulating the expression or production of Th1 cytokines (4–6) and have no effect or even augment the production of Th2 cytokines (6, 7). These properties render PDE inhibition a candidate therapy for Th1-mediated autoimmune disorders. Indeed, both nonselective as well as PDE4-specific inhibitors were effective in ameliorating disease in different experimental autoimmune encephalomyelitis models (5, 8–10) and in collagen-induced arthritis models (11, 12). However, the simple extrapolation of therapeutic efficacy from animal models to human disorders is not easily feasible (13), and therefore, the analysis of the immunomodulatory properties of PDE inhibitors on human immune cells is an important step in preclinical testing. Studies exploring the effects of PDE inhibitors in humans in vitro or in vivo are still limited. Although there is some evidence for a preferential inhibition of proinflammatory cytokines in Th1-mediated human autoimmune conditions (2, 14, 15), data from asthmatic and atopic individuals reached almost the opposite conclusion, i.e., that these drugs lead to preferential inhibition of Th2 responses (16). We therefore decided to study the effects of the selective PDE4 inhibitor rolipram and the PDE3 inhibitor cilostamide on a large number of human autoreactive and foreign Ag-reactive T cell lines (TCL) derived from MS patients and healthy individuals and asked the following questions. 1) Which biological functions (Ag-specific proliferation, cytokine production, functional Ag avidity) of human $CD4^+$ T cells are influenced by these PDE inhibitors? 2) Can these effects be induced by the concentrations of drugs achievable in vivo? 3) Is there a differential effect between PDE4 and PDE3 inhibitors and their combination? 4) Is there a differential sensitivity to the PDE inhibition between autoreactive and foreign Ag-reactive TCL and between TCL derived from MS patients vs healthy donors? Finally, 5) what are the possible explanations of the immunomodulatory effect of PDE inhibitors?

Materials and Methods

Reagents, generation of T cell lines, and proliferation assays

Rolipram (racemate of 4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone) was provided by Dr. Harald Dinter (Berlex Laboratories, Richmond, CA), Cilostamide (OPC 3689) was a gift from Dr. Vincent Manganiello Pulmonary-Critical Care Medicine Branch (PCCMB), National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). Fresh solutions of individual drugs were prepared for each experiment. The solvent for both drugs, DMSO (Sigma, St. Louis, MO), was used in 1/1000 dilution with T cell medium for the 10-μM concentration of drugs and at this concentration did not influence T cell proliferation when used as a negative control.

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[2] Abbreviations used in this paper: MS, multiple sclerosis; PDE, phosphodiesterase; MBP, myelin basic protein; TCL, T cell line.

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Myelin basic protein was prepared as previously described (17). The peptides Flu-HA$_{306-318}$ (PKYVKQNTLKLAT) and tetanus$_{830-843}$ (QYIKANSKFIGITQL) were synthesized by continuous flow, solid phase peptide synthesis on the basis of the F-moc/Bu^t strategy. Peptides were purified by HPLC, and their identities were tested using ion spray mass spectrometry.

The TCL were generated by an IL-7-modified primary proliferation assay, a method that allows the rapid expansion of Ag-specific T cells, including in vivo activated cells. Briefly, PBMC were isolated from fresh leukophereses by Ficoll density gradients and were seeded in 96-well U-bottom plates in T cell medium (IMDM, Life Technologies, Grand Islands, NY) containing 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin/streptomycin (all from BioWhittaker, Walkersville, MD), and 5% pooled human plasma at 1×10^5 cells/well, with addition of 10 ng/ml IL-7 (recombinant human IL-7; PeproTech, Rocky Hill, NJ). After 7 days (37°C and 5% CO_2), cultures were split by transferring 100 μl of each cell culture into a daughter plate, which was pulsed with [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 1 μCi/well. The incorporated radioactivity (counts per minute) was measured by scintillation counting (Betaplate, Pharmacia LKB, Piscataway, NJ) 8 h later. Proliferation of cultures with Ag (25 μg/ml MBP or 5 μg/ml peptides) was compared with proliferation of negative control wells seeded without Ag. Positive cultures (stimulation index >2 and absolute counts per minute at least 3 SDs above the average counts per minute of negative control wells) were identified on the mother plates and were periodically restimulated. The Ag specificity was confirmed at the end of the second in vitro stimulation cycle in 48-h proliferation assays as previously described (18). All blood samples were collected according to an institutional review board-approved protocol, and informed consent was obtained before the study. None of the patients received any immunomodulatory or immunosuppressive treatment within 1 mo before blood collection.

Effects of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation and functional Ag avidity

The effects of PDE inhibitors on Ag-specific proliferation, cytokine production, and functional Ag avidity were assessed during the third stimulation cycle (days 22–25 ex vivo). Each drug was used alone (in three concentrations: 0.1, 1, and 10 μM) or in combination (0.05, 0.5, and 5 μM, concentrations of individual drugs in the mixture). The selected dose range included the concentrations achievable in vivo (for rolipram, 0.09 and 0.2 μM; Dr. Claus-Steffen Stuerzebecher, Schering, Berlin, Germany, personal communication). Each condition was tested in duplicate, including negative (no Ag) and positive (Ag, no drug) controls. T cells were plated in 96-well U-bottom plates at 2×10^4 T cells/well with irradiated autologous PBMC at 1×10^5 cells/well. The Ag was added at the seeding concentration (25 μg/ml MBP and 5 μg/ml peptides) or over a wide range of Ag concentrations for the functional Ag avidity assay. Supernatants from these assays were collected after 36 h of incubation and stored frozen until analysis. For the last 8 h of incubation, cells were pulsed with [^{3}H]thymidine at 1 μCi/well, and the incorporated radioactivity was measured by scintillation counting.

Cytokine secretion

Secretion of a Th1 (IFN-γ) and a Th2 (IL-4) cytokine by Ag-specific TCL was assessed by sandwich ELISA (Cyto-Sets from BioSource International, Camarillo, CA) according to the manufacturer's recommendation. All standards and samples were tested in duplicate.

Flow cytometry (FACS) analysis of the surface expression of costimulatory molecules

Fresh PBMC (1.2×10^6 cells/ml) were seeded in bulk cultures in 48-well plate with or without rolipram (10 μM). In addition to nonstimulated cells, the effect of rolipram was assessed upon nonspecific stimulation with LPS (2.5 μg/ml) or PHA-P (PHA; 5 μg/ml; both from Sigma). After 12-h incubation cells were washed with wash buffer (Dulbecco's PBS with 1% heat-inactivated FCS and 0.1% sodium azide) and incubated with fluorescein-, PE-, or Cy-Chrome-conjugated Ab (HLA-DR,DP,DQ-FITC, CD19-FITC, CD14-FITC and -PE, CD80-FITC and -PE, CD86-FITC and -PE, and CD3-Cy-Chrome; all from PharMingen, San Diego, CA) at saturating concentrations for 30 min on ice, then washed three times and analyzed (FACScan, Becton Dickinson, CA) using Cell-Quest software. Isotype-matched mouse IgG negative controls were used for each staining. Monocytes were gated based on the size characteristics (forward and side scatter), and expression of CD14. Lymphocytes were identified by the size characteristics and differentiation between T and B lymphocytes was based on the expression of CD3 and CD19 molecules, respectively. Five thousand cells in the gated population were analyzed per sample.

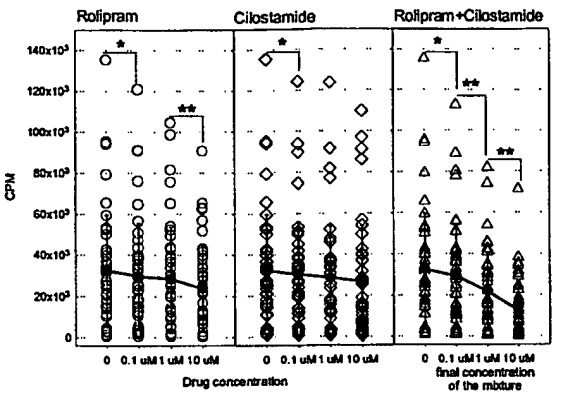

FIGURE 1. Effect of PDE inhibitors on Ag-specific proliferation of TCL. A total of 47 TCL was stimulated with specific Ag without or with three increasing concentrations of rolipram and cilostamide. For the combination of rolipram and cilostamide, each drug was used in concentrations of 0.05, 0.5, and 5 μM, respectively. Results are depicted as counts per minute. Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$). Only rolipram and the combination of both drugs had a dose-related inhibitory effect on Ag-specific TCL proliferation. The combination of rolipram and cilostamide was the most potent, demonstrating a synergistic effect of individual drugs. The differences among three treatment modalities reached statistical significance ($p < 0.05$, by repeated measures ANOVA analysis).

Statistical analysis

The data were analyzed by a commercial software package (Sigma-Stat, SPSS, Chicago, IL). The effects of the drugs on biological functions of TCL was evaluated by one-way repeated measures ANOVA or, if normality failed, by Friedman's repeated measure analysis on ranks. Statistically significant differences from repeated measures ANOVA were further analyzed by the Student-Newman-Keuls test, with $p < 0.05$ as a cut-off for statistical significance. The effect of rolipram on costimulatory molecules was assessed by the Mann-Whitney rank-sum test.

Results

Effect of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation

We examined the effects of rolipram and cilostamide on the proliferation of 47 Ag-specific TCL. Both drugs inhibited Ag-driven TCL proliferation, but only rolipram and the combination of both drugs expressed statistically significant inhibitory effects in a dose-dependent manner (Fig. 1). The inhibition by rolipram (8.8–32% inhibition) was more pronounced than that by cilostamide (6.31–22.61% inhibition). Combinations of both drugs (1/1 ratio and half molar concentration of each drug (0.05, 0.5, and 5 μM in the final mixture) proved to be most efficient in inhibiting TCL proliferation (10.34–61.05%), exceeding the additive effects of individual drugs. The observed differences between drugs and their combination were statistically significant ($p < 0.05$, by repeated measures ANOVA). Because the above characteristics of the drug combination indicated synergistic effects, we have quantified the degree of synergism between rolipram based on the modified Berenbaum equation (19):

The degree of synergism between drugs A and B is equal to $1/R$, where $R = [(IC_{20}\text{ drug A} + B)/(IC_{20}\text{ drug A})] + [(IC_{20}\text{ drug B} + A)/(IC_{20}\text{ drug B})]$.

Based on the proliferation data in Fig. 1 we have estimated the IC_{20} (the concentration of each drug that leads to 20% inhibition of TCL proliferation; this was substituted for IC_{50}, as neither rolipram nor cilostamide reached 50% of inhibition) from the dose-response curves and calculated $R_{\text{rolipram+cilostamide}} = 0.1123$. The

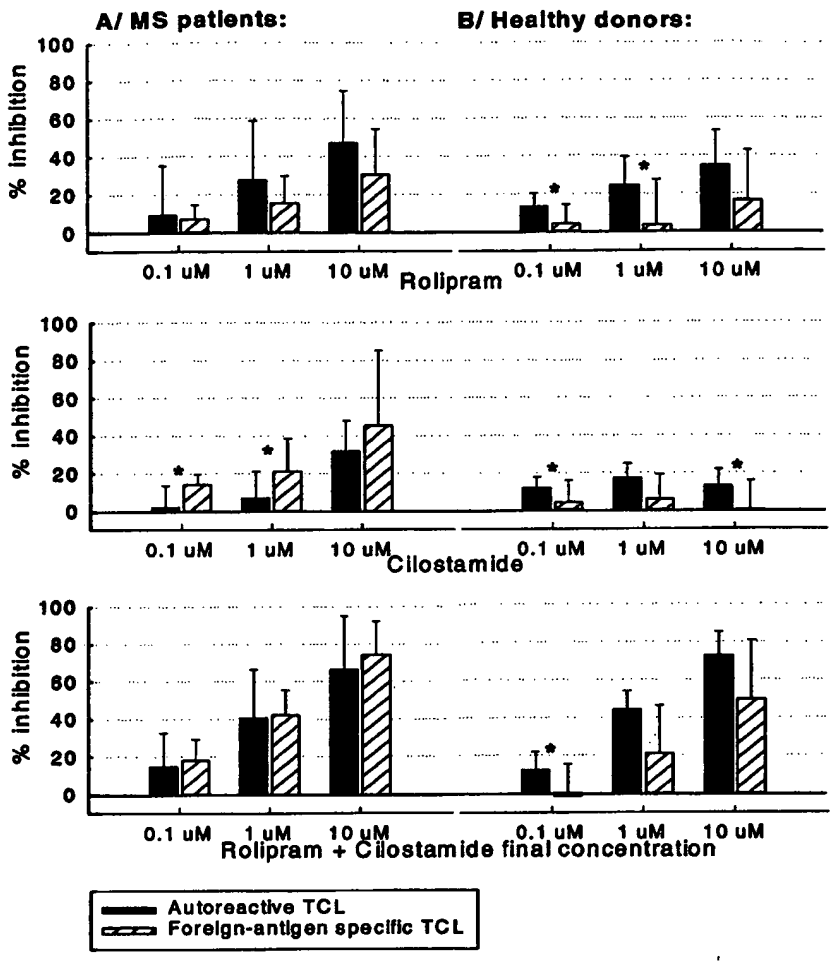

FIGURE 2. Effects of PDE inhibitors on proliferation of TCL stratified based on their origin (derived from MS patients vs healthy donors) and selecting Ag (autoreactive vs foreign-Ag specific). Upon stratification of the proliferation data with respect to the origin of the TCL and the type of selecting Ag, a hierarchy of the sensitivity to PDE inhibition emerged; MS-derived TCL were more sensitive than healthy donor (HD)-derived TCL. A similar differential sensitivity to PDE4 vs PDE3 inhibition was observed in the analysis of autoreactive and foreign Ag-specific TCL within each population group. Statistically significant differences are indicated ($p < 0.05$, by Friedman's repeated measures analysis on ranks).

degree of synergism between rolipram and cilostamide is $1/R = 8.9$. (Values <1 indicate antagonism, 1 indicates additivity, and values >1 indicate synergism.)

Comparison of the susceptibility to PDE-4 and -3 inhibition by autoreactive and foreign Ag-specific TCL and by TCL derived from MS patients and healthy donors

When the inhibitory effect of PDE inhibitors was assessed after stratification of TCL based on their origin and the type of the selecting Ag (TCL derived from MS patients (26 TCL, 17 autoreactive, 9 foreign-Ag-specific) vs. TCL derived from healthy donors (21 TCL, 5 autoreactive, 16 foreign-Ag-specific)), two interesting observations emerged (Fig. 2). 1) The MS-derived TCL were more susceptible to the effect of PDE inhibitors than were the healthy donor-derived TCL ($p < 0.05$ for 10 μM rolipram, 10 μM cilostamide, and 0.1 μM and 1 μM concentrations of drug mixture; Friedman's repeated measures analysis on ranks). 2) Also, there appeared to be a differential sensitivity of autoreactive TCL and foreign Ag-specific TCL to the effects of PDE4 and PDE3 inhibition. Autoreactive TCL were inhibited by rolipram to a greater extent than foreign Ag-specific TCL ($p < 0.05$, through the tested concentration range of rolipram, Friedman's repeated measures analysis on ranks). Only stratified data are depicted in Fig. 2. Due to the decreased power of a comparison of divided data into individual patient groups, not all differences reached statistical significance ($p < 0.05$, by Friedman's repeated measures analysis on ranks); these are marked with an asterisk.

Effect of PDE inhibitors on cytokine production by Ag-specific TCL

We evaluated the effects of PDE inhibitors on Ag-driven production of two cytokines, IFN-γ (prototypic Th1 cytokine) and IL-4 (prototypic Th2 cytokine; Fig. 3). Although a significant variability was noted among individual TCL, the average effect of PDE4 inhibition by rolipram and of PDE4 and PDE3 inhibition by the combination of drugs on IFN-γ production was inhibitory. Rolipram (12.26–27.8% inhibition) and the combination of both drugs (16.07–46.3%) inhibited IFN-γ synthesis in a dose-dependent manner. The effect of cilostamide was mild and did not reach statistical significance for any concentration (4.47–11.27% inhibition). Differences among the drugs were again statistically significant.

Neither rolipram nor the combination of rolipram and cilostamide had a statistically significant effect on IL-4 production (inhibition ranging from -0.62 to 1.69% throughout the concentration range). Cilostamide had a mild inhibitory effect on IL-4 production at 0.1 μM, whereas a high concentration (10 μM) significantly enhanced IL-4 production (-11.39% inhibition at 10 μM concentration). Overall, the effect of PDE4 and PDE3 inhibitors on IFN-γ production paralleled those on proliferation, with the drug combination having synergistic effects. The IL-4 production was largely unaffected despite the demonstrated significant inhibition of TCL proliferation at the drug concentrations tested.

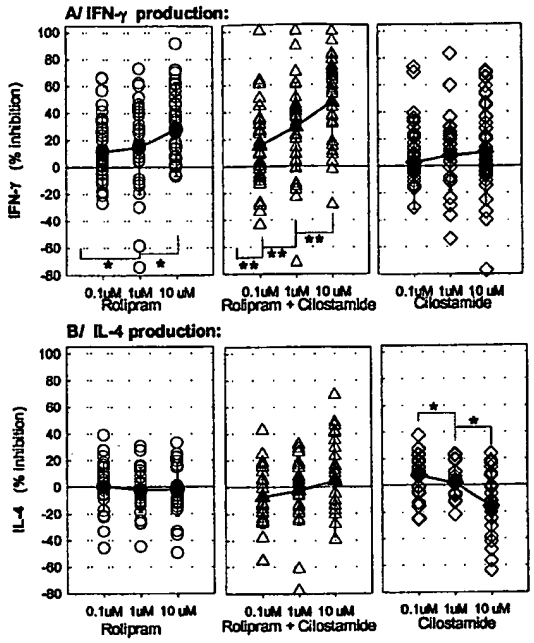

FIGURE 3. Effect of PDE inhibitors on cytokine production of Ag-specific TCL. The effects of rolipram, cilostamide and their combination on Ag-specific production of IFN-γ and IL-4 was assessed in parallel to the proliferation. Results are depicted as the percent inhibition of cytokine production for each concentration of drug compared with control cultures (no drug added). Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$). Although all TCL tested in proliferation assays produced IFN-γ and are included in the analysis, only 9 of 26 MS-derived TCL and 17 of 21 healthy donor-derived TCL produced IL-4; therefore, only these 26 TCL could be included in the analysis of the effect of PDE inhibitors on IL-4 production. Only rolipram and the combination of rolipram with cilostamide inhibited the production of IFN-γ significantly compared with control samples. This inhibition paralleled the effect on TCL proliferation and was clearly strongest for the combination of both drugs. Neither rolipram nor the drug combination has a significant effect on IL-4 production. A high concentration (10 μM) of cilostamide enhanced IL-4 production despite the demonstrated inhibitory effect on proliferation. Differences between individual drugs were statistically significant ($p < 0.05$).

Effect of PDE inhibitors on the functional Ag avidity of TCL

To gain a better understanding of the mechanism of the PDE4 and PDE3 inhibitor-induced bias of immune responses from a Th1 toward a Th2 phenotype, we decided to study their effects on the major components of T cell priming. Apart from the influence on the cytokine milieu, which was supported by the above experiments, we wanted to address the question of whether these drugs influence the Ag dose needed for T cell activation, i.e., antigen avidity, and costimulatory signals delivered by APC. First, to address the question of whether the magnitude of the immunomodulatory effect of PDE inhibitors on TCL varies depending on the dose of Ag, we exposed a subgroup of TCL (15 TCL; selected as a representative sample based on their phenotype, variable susceptibility to PDE inhibition, and reactivity to either autoantigen or environmental Ag; Table I) to a 1-μM concentration of the individual drugs over a wide range of Ag concentrations. The results of this functional Ag avidity assay for three TCL (of Th1, Th0, and Th2 phenotypes) are summarized in Fig. 4. None of the autoreactive or foreign Ag-specific TCL that we generated for this project expressed a clear Th2 phenotype. We therefore included a Th2-like TCL specific for copolymer-1 (Cop-1), an approved immunomodulatory drug for MS. The TCL Th1:MBP and Th0:Flu-HA were representative of all other tested TCL and illustrate the above-mentioned differential effect of PDE inhibition on autoreactive vs foreign Ag-reactive TCL. All examples also demonstrate the hierarchy of immunomodulation between individual drugs (cilostamide < rolipram < rolipram + cilostamide). For MBP-specific Th1-TCL, a combination of PDE4 and PDE3 inhibition resulted in almost 100% inhibition of proliferation and IFN-γ production. For Flu-HA-specific Th0-TCL, the inhibition of proliferation and IFN-γ production was much less pronounced (~50% with the combination of drugs for each Ag concentration), and the production of IL-4 was either unaffected or even enhanced. For Cop-1-specific Th2-TCL, the individual drugs had no effect on Ag-specific proliferation or IL-4 production. Overall, the magnitude of the effect of PDE inhibitors on individual TCL was similar through the tested Ag concentration range. However, as demonstrated in Table I, the PDE inhibition had a mild, but statistically significant, effect on the EC_{50} (concentration of Ag that leads to 50% maximal proliferation) of individual TCL ($p = 0.009$, Friedman's repeated measures analysis on ranks).

Table I. *Effect of PDE inhibitors on TCL Ag avidity (EC_{50})*

TCL	Ag	TCL Origin	Phenotype	EC_{50} (μg/ml)			
				No drug	Cilostamide	Rolipram	Rolipram + cilostamide
1	Flu-HA	MS	Th1	0.070	0.070	0.080	0.200
2	Flu-HA	MS	Th1	0.070	0.200	0.200	0.200
3	Flu-HA	MS	Th1	0.009	0.010	0.030	0.500
4	Flu-HA	MS	Th0	0.003	0.003	0.100	0.100
5	Flu-HA	MS	Th0	0.008	0.010	0.100	0.100
6	MBP	MS	Th1	0.050	0.070	0.070	0.100
7	MBP	MS	Th1	10.000	10.000	—[a]	—[a]
8	MBP	MS	Th1	2.000	3.000	2.000	3.000
9	MBP	MS	Th1	1.500	1.500	1.500	1.400
10	MBP	HD	Th1	20.000	20.000	20.000	30.000
11	MBP	MS	Th0	3.000	3.000	10.000	—[a]
12	MBP	MS	Th0	1.000	1.500	1.000	1.000
13	MBP	HD	Th0	25.000	25.000	25.000	25.000
14	MBP	HD	Th0	3.000	3.000	4.000	3.000
15	Cop-1	HD	Th2	3.000	3.000	3.000	3.000
Total	Mean ± SD			4.58 ± 7.77	4.69 ± 7.73*	4.79 ± 8.02*	5.20 ± 10.02*

[a] 100% inhibition of proliferation.
*, $p < 0.05$; Friedman's repeated measures analysis on ranks with Student-Newman-Keuls test.

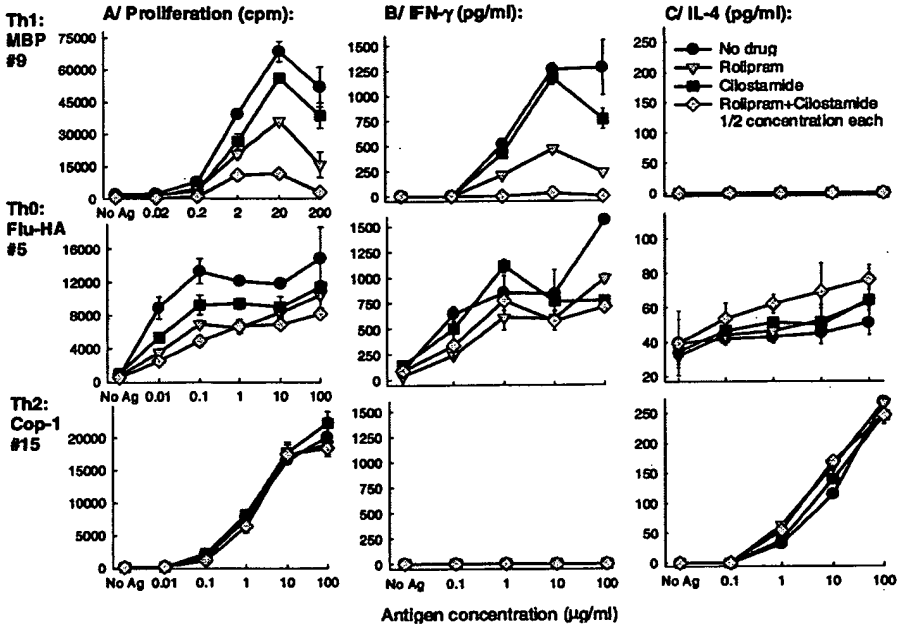

FIGURE 4. Effect of PDE inhibitors on functional Ag avidity. A total of 15 TCL were tested in Ag dose-response assays in the presence of 1 μM of drug and a wide Ag concentration range. Only three TCL are depicted in this figure. Th1:MBP (TCL 9 in Table I) and Th0:Flu-HA (TCL 5 in Table I) were selected as representative TCL for 14 MBP- or Flu-HA-specific TCL tested. The Th2:Cop-1 (TCL 15 in Table I) TCL was added for this assay only, as none of the 47 MBP- or Flu-HA-specific TCL tested in previous experiments expressed a clear Th2 phenotype. The EC_{50} (Ag concentration leading to 50% maximal proliferation of TCL) values were calculated from these dose-response curves for each TCL and are summarized in Table I. The TCL depicted in this figure demonstrate the hierarchy of potency of the immunomodulatory effect between individual drugs. The combination of rolipram and cilostamide had the strongest inhibitory effect on T cell proliferation and IFN-γ secretion, while having no effect or enhancing the production of IL-4. The differential susceptibility to PDE inhibitor-mediated immunomodulation on TCL was noted based on their cytokine phenotype, with Th1-TCL being more susceptible than Th0 or Th2-TCL. Overall, the magnitudes of the effects of PDE inhibitors on individual TCL were similar through the tested Ag concentration range.

Effect of rolipram on the surface expression of costimulatory molecules

Finally, we wanted to assess the effect of PDE inhibition on the third important component of T cell priming, the costimulatory signal. We studied the effect of rolipram on the surface expression of costimulatory molecules of human PBMC in the resting and in the activated state. Freshly isolated PBMC were seeded ex vivo with or without rolipram, in a resting state or upon activation with nonspecific stimuli (PHA or LPS). After 12 h we analyzed these cells by three-color flow cytometry. Rolipram consistently downregulated the surface expression of MHC class II both in the resting state and after stimulation with PHA and LPS on monocytes (Fig. 5) and T and B lymphocytes (data not shown). After 12-h stimulation of PBMC with LPS, rolipram-treated cultures had decreased surface expression of CD80 ($p < 0.001$, by Mann-Whitney rank-sum test) and increased surface expression of CD86 ($p = 0.008$; Fig. 5). Similar changes in the expression of costimulatory molecules were observed on B and T lymphocytes or after stimulation with PHA (data not shown).

Discussion

In this article we present a detailed analysis of the effects of the selective PDE4 inhibitor rolipram and the PDE3 inhibitor cilostamide on human immune cells to determine the potential of these drugs for the treatment of human Th1-mediated autoimmune disorders. Consistent with the data obtained from animal models (5, 10, 11), we demonstrated the predominant inhibitory effect of PDE inhibitors on Th1-mediated immune responses in humans. To address the effect of PDE inhibitors on the cytokine phenotype of human T cells, we focused our experiments on short term TCL and performed our analysis on days 22–25 ex vivo to avoid possible artifacts of long term culture. Our data on the modulation of Th2-TCL by PDE inhibitors are limited, but in agreement with our cytokine data from Th0-TCL (no effect, or even induction of IL-4) and with data from different experimental systems (7, 20, 21); we found no inhibition of Ag-specific TCL proliferation and IL-4 production in these TCL.

Few studies have addressed the question of the effect of PDE inhibitors on human immune cells. A recent study from our laboratory examined the expression of PDE4 and PDE3 enzymes in autoreactive MBP-specific TCL (2) and demonstrated that these two families account for the vast majority of PDE enzymatic activity in these cells. This is consistent with our current data, demonstrating a significant inhibition of proliferation at the highest concentration of the drug combination (5 μM of both rolipram and cilostamide) for virtually all tested TCL. Another study analyzed the influence of rolipram on the functional characteristics of nine MBP-specific TCL, five derived from MS patients and four from healthy donors (15), and raised the issue of a differential susceptibility of individual TCC to the immunomodulatory influence of rolipram. Although rolipram inhibited TNF-α and -β as well as IL-10 production by TCL, the effect on other cytokines (IFN-γ, IL-4, and IL-13) was inconsistent and did not reach statistical significance. Moreover, Essayan et al (16). suggested a higher susceptibility to PDE4 inhibition by Th2-TCL compared with Th1-TCL in a limited number of Th1 and Th2 TCL (total of four) derived from atopic and asthmatic patients. Although it is difficult to compare the data derived from different experimental systems, the reported inhibition of Th1-TCL derived from these asthmatic patients did not reach the magnitude of the typical inhibition of MS-derived Th1 TCL observed in our laboratory using the same concentrations of drugs. Because asthmatic individuals and MS

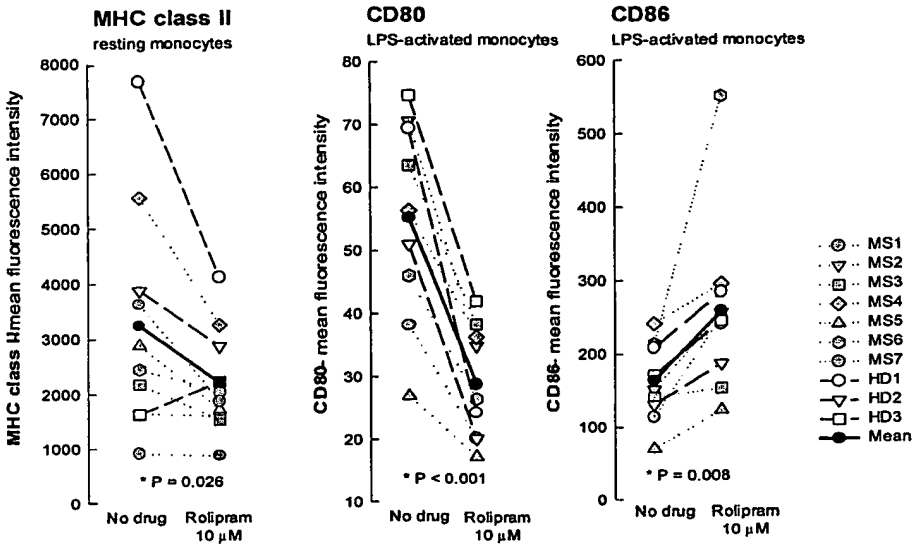

FIGURE 5. Effect of rolipram on the expression of costimulatory molecules by APC. The surface expression of MHC class II and costimulatory molecules CD80 and CD86 on monocytes and B and T lymphocytes was assessed by triple-staining, flow cytometric analysis. Resting or nonspecifically activated (LPS or PHA) PBMC were incubated for 12 h in bulk culture with or without rolipram and analyzed by flow cytometry. Only the effect on monocytes (gated based on size characteristics and CD14 expression), both rested and activated by LPS, is depicted in this figure, but similar results were obtained upon activation by PHA or when gating of B or T lymphocytes. Rolipram down-regulates MHC class II expression on both resting and activated cells, while it down-regulates CD80 and up-regulates CD86 expression upon activation with either nonspecific stimulus. These changes were observed in both MS patients and healthy donors.

patients have biased immune responses toward opposite Th phenotypes as compared with unbiased responses of healthy donors, we asked whether the explanation for these controversial data from human TCL lies in the differential susceptibility to PDE inhibition between these different patient groups. This hypothesis prompted us to examine the effect of PDE inhibition on large numbers of TCL, derived from both healthy donors and MS patients, and with specificity for two Ags, the autoantigen MBP and the classical foreign recall Ags Flu-HA or tetanus (22). Indeed, we were able to demonstrate a higher susceptibility to PDE inhibition by MS-derived compared with healthy donor-derived TCL. This finding has several important implications. It explains how the same therapeutic agent could be considered for the treatment of disorders with potentially different pathogenesis (Th1-mediated autoimmune disorders vs. Th-2-mediated asthma and atopic dermatitis). We and others recently demonstrated (23, 48) that the immune system in MS patients is in a dysregulated state characterized by an overshooting Th1 response not only to autoantigens, but also to common environmental pathogens. A similar dysregulated state, this time toward Th2 responses, is likely to exist in asthmatic or atopic individuals (24). If such a dysregulation involves abnormalities in the cAMP second messenger system, it would render TCL derived from these individuals more susceptible to the effects of PDE inhibition, thus at least in part explaining the controversies between results obtained from MS and asthmatic patients. Several reports in the literature indicate that this may be the case. Patients with MS, rheumatoid arthritis, or lupus were found to have low intracellular cAMP levels (25, 26), decreased expression and activity of G protein-coupled receptor kinases (27), or deficient type I cAMP-dependent protein kinase A activity (28, 29). Similar abnormalities in cAMP signaling were suggested in asthmatic patients (30–32).

It is more difficult to explain the suggested differential susceptibility to PDE inhibition between autoreactive and foreign Ag-reactive TCL. We did not find any significant skewing in the cytokine profiles between these two types of TCL that would account for the observed differential effect. This effect is not mediated by the need for antigenic processing of MBP compared with Flu-HA and tetanus peptides, because it was shown that MBP presentation by HLA-DR molecules does not require processing (33) and because we have noted a similar effect of PDE inhibition on seven MBP-specific TCL stimulated with the peptide epitope (data not shown). The two likely explanations are that either the signal delivered by the autoantigen may be qualitatively different (partial agonist vs full agonist signal) or the dysregulation in the cAMP system is more pronounced in autoreactive T cells. We are currently studying this issue in detail. Considering the therapeutic use of PDE inhibitors, the observed higher susceptibility of autoreactive TCL to PDE4 inhibition may widen the therapeutic window in the treatment of autoimmune disorders without inducing general immunosuppression.

To explore the possible cause of the PDE inhibitor-induced bias from Th1 to Th2 phenotype, we decided to study the influence of these drugs on T cell priming. There are three major components contributing to T cell priming, which may influence the phenotype of the primed T cell: the cytokine milieu, the dose and character of the Ag, and the costimulatory signal. It was previously demonstrated by us and others that selective PDE4 inhibitors or nonselective PDE inhibitors decrease the secretion or expression of proinflammatory cytokines by human mononuclear cells, favoring the cytokine milieu at the time of Ag presentation toward an anti-inflammatory Th2 phenotype (6, 14, 34, 35). However, the influences of PDE inhibition on the other components of T cell priming conditions were unknown. First, we studied the PDE inhibitors in Ag dose-response assays, exploring their effects on the dose of Ag required for T cell activation. The results of these studies indicate that PDE inhibitors have two different effects on the activation of TCL; they inhibit TCL proliferation regardless of the Ag dose (Fig. 4). On the other hand, the effect of PDE inhibitors on the EC_{50} indicates that a higher Ag dose is necessary for the activation of TCL under their influence. These data suggest that PDE inhibitors have complex inhibitory effects on T cell activation, most likely by influencing both proximal Ag-responsive events of T cell signaling

(36) as well as components of more downstream machinery involved in T cell effector functions. The exact mechanisms of this effect are currently under investigation in our laboratory. The demonstrated influence of PDE4 and -3 on the Ag dose required for T cell activation together with the data from the literature showing that high Ag doses skew the developing immune response toward a Th1 phenotype, whereas low doses of Ag skew the response toward a Th2 phenotype (37) adds another mechanism for the observed effect of these drugs on Th1/Th2 paradigm.

Next, we wanted to assess the influence of rolipram on the third component of T cell priming, the costimulatory signals. It has been suggested that costimulation by CD80 preferentially drives the T cell differentiation toward Th1 responses, whereas CD86 costimulation biases T cell priming toward Th2 responses (38). Although some concerns were raised regarding the general validity of this dichotomy of the roles of CD80 and CD86 (39), several reports indicate that the CD80/CD86 costimulatory system is altered in MS patients. Specifically, higher numbers of $CD80^+$ B lymphocytes in the cerebrospinal fluid (40, 41) increased serum levels of $CD80^+$ lymphocytes in patients during MS exacerbation (42), and low expression of CD86 on cerebrospinal fluid T cells (41) have been reported in patients with MS. Our data indicate that rolipram down-regulates CD80 expression and up-regulates CD86 expression on monocytes and B and T lymphocytes upon nonspecific activation with PHA or LPS. The likely explanation for this observation is the differential kinetic of induction of these costimulatory molecules on APC; CD80 is expressed later than CD86; therefore, rolipram may be preventing the switch from CD86 to CD80 expression, an issue that merits further study. We also demonstrated that rolipram down-regulates MHC class II expression, both in resting conditions and after induction by proinflammatory signals. This finding is in agreement with the observation in a murine system, where increases in intracellular cAMP inhibit the IFN-γ-mediated induction of class II MHC genes (43). Together, these changes in the costimulatory profile on APC favor T cell priming from Th1 toward a Th0 or Th2 phenotype and may limit the effective presentation of autoantigen in inflammatory MS lesions. Indeed, rolipram was shown to reduce the number of IFN-γ-secreting cells upon priming of human mononuclear cells in bulk cultures to the autoantigen MBP, while the numbers of IL-4- or IL-10-secreting cells were unaffected (44).

The above data demonstrate a favorable drug profile of PDE4 and PDE4 combined with PDE3 inhibitors for the treatment of Th1-mediated autoimmune disorders; however, the question remains of whether this immunomodulatory effect is expressed within a concentration range that is achievable in humans in vivo. The concentrations of rolipram achievable in healthy volunteers following the administration of 0.75 mg three times daily and 1.5 mg three times daily were 24 and 53 ng/ml (0.09–0.2 μM; Dr. Claus-Steffen Stuerzebecher, unpublished observations) Our data, summarized in Fig. 6, indicate that rolipram and especially the combination of rolipram and cilostamide have a mild immunomodulatory effect at the concentration of 0.1 μM. However, due to the immunomodulation at multiple levels (influence on T cell priming conditions, Ag-specific proliferation, and cytokine production) the in vivo effect is likely to be more prominent. The combination of PDE4 and PDE3 inhibitors expresses a high degree of synergism that is, to our experience, achievable even at much lower concentrations of cilostamide in the final mixture (data not shown). Therefore, the combination of PDE4 inhibitors with relatively small amounts of PDE3 inhibitors may represent a way to broaden the therapeutic window in the treatment of human disorders and should be considered for future drug development. The molecular mechanism of this synergy is not known. It has been

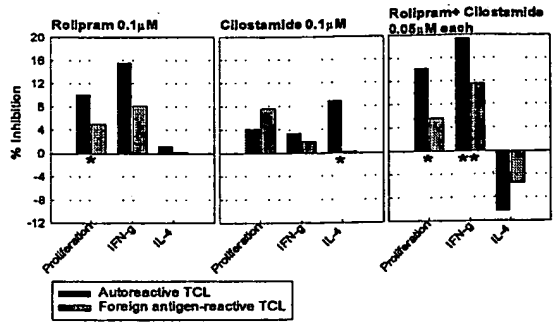

FIGURE 6. Immunomodulatory effect of PDE inhibitors within in vivo achievable concentration ranges. The concentration of rolipram that can be achieved in humans in steady state without major side effects ranges from 0.09 to 0.2 μM. At a concentration of 0.1 μM, PDE inhibitors express a mild, but multilevel, immunomodulatory profile by suppressing Ag-specific proliferation and IFN-γ production preferentially of autoreactive TCL, while not affecting, or even enhancing, IL-4 production. This immunomodulation is strongest for the combination of rolipram and cilostamide, demonstrating the synergistic effect. A statistically significant effect compared with control cultures is indicated (*, $p < 0.05$; **, $p < 0.01$).

reported that PDE3 inhibitors alone have little effect on the total intracellular cAMP levels, and they do not further enhance the cAMP accumulation induced by rolipram (45). However, it has been suggested, that PDE3 (predominantly localized to the particulate cellular fraction) and PDE4 (predominantly cytosolic) may regulate different pools of cAMP (46, 47). It is conceivable that intracellular signaling can partially adapt to the effects of PDE4 inhibition by diverting critical pathways blocked by high cytosolic cAMP concentrations and activation of PKA to the alternative pathways, which, in turn, may be affected by PDE3 inhibition. Elucidating the molecular mechanism of this synergy between PDE4 and PDE3 inhibition will enhance our understanding of cAMP second messenger signaling.

Well-designed therapeutic trials supported by immunological studies should not only provide more definite information about the therapeutic potential of PDE inhibitors in autoimmune disorders, but also broaden our understanding of the immunopathogenesis and the potential differences among individual human Th1-mediated autoimmune diseases.

Acknowledgments

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Review

Versatile effects of sildenafil: recent pharmacological applications

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Abstract:
Sildenafil is a phosphodiesterase-5 (PDE5) inhibitor and is predominantly used in the treatment of erectile dysfunction. While maintaining an excellent safety and tolerability profile in the management of erectile dysfunction, sildenafil also provides a prolonged benefit in various other diseases. Sildenafil has been shown to have a potential therapeutic efficacy for disorders related to the central nervous system and pulmonary system. In the central nervous system, it exerts its neuroprotective effects in multiple sclerosis and has a significant memory enhancing action. Sildenafil also significantly enhances neurogenesis. Several lines of evidence indicate that targeting PDE5 with sildenafil offers novel strategies in the treatment of age-related memory impairment. Guanylate cyclase/cGMP/protein kinase G pathway or glutamate/nitric oxide/cGMP pathway appears to mediate memory enhancing effects. Some of the positive cognitive features of sildenafil therapy are likely attributable to the mechanisms reviewed here. Sildenafil has been shown to reduce pulmonary hypertension and alleviate pain in animals and humans. The present review primarily focuses on the various pharmacological effects of sildenafil with regard to its influence on the nervous and pulmonary system.

Key words:
sildenafil, neurogenesis, memory enhancement, antinociception, pulmonary hypertension

Abbreviations: AD – Alzheimer's disease, Akt – protein kinase B, AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, CaM KII – calmodulin-dependent protein kinase II, cGMP – cyclic guanosine monophosphate, cGK – cGMP-dependent protein kinase, GC – guanylate cyclase, sGC – soluble guanylate cyclase, GSK-3 – glycogen synthase kinase 3, GTP – guanosine triphosphate, LTP – long-term potentiation, PKG – protein kinase G, NO – nitric oxide, NMDA – N-methyl-D-aspartate, PDE5 – phosphodiesterase-5

Introduction

Sildenafil is the first oral drug approved by the United States Food and Drug Administration for the therapeutic treatment of erectile dysfunction. Sildenafil (a chemical compound designated as UK-92,480) was initially synthesized by pharmaceutical scientists

working at Pfizer's research facility in Kent, United Kingdom. The focus of research by the scientists at Pfizer was to treat hypertension and angina pectoris. However, the phase I clinical trials did not yield a significant antihypertensive effect and sildenafil had little therapeutic potential for the treatment of angina. Interestingly, sildenafil exhibited a different pharmacological effect in these patients, marked penile erection [59]. This unexpected pharmacological finding resulted in patenting sildenafil in 1996 by Pfizer. Later, sildenafil was approved by the Food and Drug Administration in 1998 for use in erectile dysfunction. Thus, the miracle drug sildenafil became the first oral drug approved for the therapeutic treatment of erectile dysfunction in the United States and the sale exceeded 1 billion dollars in the period of 1999–2001. Sildenafil citrate is a water soluble aromatic compound and its main pharmacological action is through the inhibition of phosphodiesterase-5 (PDE5) in the corpus cavernosum which contains most of the blood in the penis during erection [24, 55, 94, 112].

PDE5 belongs to an important family of proteins that regulates the intracellular level of cyclic guanosine monophosphate (cGMP). There are eleven different types of phosphodiesterases which are distributed throughout the body [59]. Phosphodiesterases hydrolyse cyclic nucleotides and, therefore, are involved in second messenger signaling pathway [24]. Among the various types of phosphodiesterases, only three selectively hydrolyze cGMP relative to cAMP. PDE5 hydrolyzes cGMP and is found in several parts of the body such as the lungs, platelets, various forms of smooth muscle and several brain regions [11, 122]. Sildenafil structurally resembles the guanosine base of cGMP and the 3-substituent extension fills a space in the enzyme active site occupied by ribose [119]. Sildenafil selectively inhibits PDE5 and increases the level of cGMP leading to beneficial effects in targeting some organs (Fig. 1). Interestingly, several lines of recent evidence indicate that sildenafil may offer novel strategy in the therapeutic treatment of age-related memory impairment, pain, pulmonary hypertension and multiple sclerosis. This review endeavors to provide an overview of such studies, and includes animal findings and potential mechanisms that may explain the pharmacological effects.

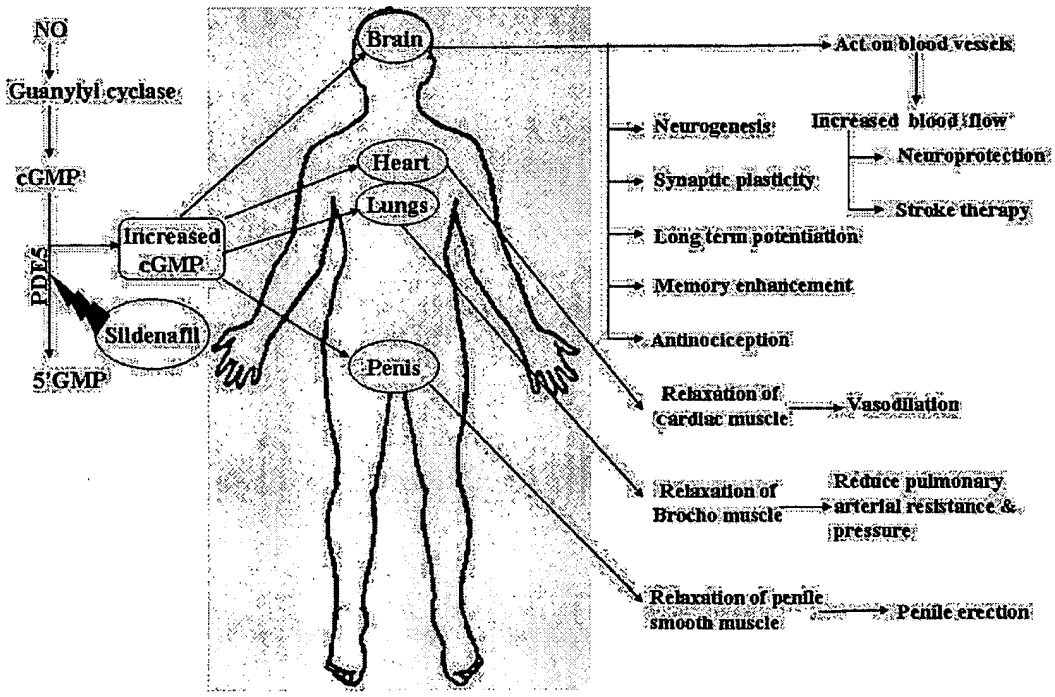

Fig. 1. Role of cGMP in various pharmacological functions in the body: effect of sildenafil on cGMP

Sildenafil and erectile dysfunction

Erectile dysfunction is an intricate physiological and psychological process which is influenced by various factors [4]. Erection is mainly initiated by visual, olfactory, imaginary stimuli and recruitment of penile afferents. Production of nitric oxide (NO) and cGMP is reduced with age and causes erectile dysfunction in many cases [115]. With regard to the psychological aspect, erectile dysfunction significantly affects the quality of life, such as objectively measured decreases in physical satisfaction, emotional satisfaction, and general happiness of a patient [79]. Erectile dysfunction is associated with feelings of isolation, low self-esteem and depression [110]. The factors that significantly affect sexual performances are environmental influence, cultural factors, gender dynamics, availability of partners and physical setting [95]. It is estimated that 20 to 30 million American men suffer from erectile dysfunction. Erectile dysfunction affects nearly 150 million men worldwide and the number is expected to more than double over the next 25 years [9]. Around the world, patients with this dysfunction usually try various natural products and other local remedies for better performance. Practitioners of Ayurvedic (ancient Indian medicine) and Chinese medicine, African herbalists and traditional healers from a wide range of cultures around the world have many centuries of knowledge regarding the use of medicines of plant origin to treat a wide variety of sexual dysfunction. Certain botanicals have been identified as having significant aphrodisiac properties and the ability to improve sexual functioning and overall systemic health. Recent studies have shown that certain botanicals have the ability to improve peripheral and systemic blood flow and to act as vasodilators, leading to increased blood flow to the penis and erectile tissue and, therefore, augment the sexual pleasure and performance [118]. There are numerous problems associated with the use of botanicals with regard to its quality, dose and dosage forms. Adverse effects and long-term toxic response of these botanicals have not been well characterized in animals and humans.

Patients with erectile dysfunction responded positively to the current pharmacological drug (synthetic) therapy. Drug therapies are based on their ability to substitute, partially or completely, the degenerated or faulty endogenous mechanisms that control penile erection. Most drugs have a direct action on penile tissue facilitating penile smooth muscle relaxation, including prostaglandin E1, NO donors, phosphodiesterase inhibitors, and alpha-adrenoceptor antagonists. Dopamine receptors in central nervous centers that participate in the initiation of erection have also been targeted for the treatment of erectile dysfunction. Sildenafil, orally administered PDE5 inhibitor, has become a first-line treatment option for erectile dysfunction. Several recent reviews focused on the beneficial effect of regular PDE5 inhibitor administration on the improvement of erectile function and the mechanism of drug action [24, 52, 55, 94, 112, 115]. Three PDE5 inhibitors (sildenafil, tadalafil, and vardenafil) in a range of doses are available [94]. Sildenafil is a potent inhibitor of cGMP specific PDE5 which is responsible for degradation of cGMP in the corpus cavernosum [17]. Inhibition of PDE5 reduces the degradation of cGMP which allows erectile function to occur by relaxation of penile smooth muscle. Sildenafil during the last 8–9 years has been prescribed by more than million physicians around the globe. Sildenafil combats sexual dysfunction caused by diverse etiology or by such different factors as drugs, psychological barriers, aging or induced by diseases like diabetes mellitus, Parkinson's disease, depression and renal failure.

Sildenafil and pulmonary hypertension

Pulmonary hypertension occurs in the major pulmonary artery that carries blood from the right ventricle of the heart to the lungs. The high blood pressure in the lungs is referred to as pulmonary hypertension. In the lungs, when the smaller blood vessels become more resistant to blood flow, the right ventricle is under stress to pump sufficient blood to the lungs. Pulmonary hypertension is a rare blood vessel disorder of the lung which may be defined as a pulmonary artery systolic pressure greater than 30 mmHg or a pulmonary artery mean pressure greater than 20 mmHg. Pulmonary hypertension can be classified as primary and secondary hypertension. The cause of primary pulmonary hypertension is not clearly understood. However, the secondary pulmonary hypertension may be due to pulmonary, cardiac and extrathoracic malfunction. Cor pulmonale may lead to various disor-

ders of the respiratory system. Pulmonary hypertension usually paves the way to cor pulmonale. The estimated prevalence of primary pulmonary hypertension is 1–2 cases/million. The prevalence of pulmonary hypertension is about 1.7 times higher in women than in men. However, in children pulmonary hypertension is seen equally often in both sexes but this is altered after puberty. Primary pulmonary hypertension mostly develops between 20 to 40 years of age and has no racial preference [100]. Pain in the chest, shortness of breath with minimal exertion, fatigue, dizzy spells and fainting are the main symptoms of pulmonary hypertension. Pulmonary hypertension can also be induced primarily by breathing disorders like emphysema and bronchitis. The other fewer causes may include scleroderma, systemic lupus erythematosus, congenital heart disease, pulmonary thromboembolism, HIV infection, liver disease, diet and drugs, like fenfluramine and dexfenfluramine.

Pulmonary hypertension is often not diagnosed properly and is thus advanced to a critical state by the time it is properly diagnosed. Due to improper disease management, pulmonary hypertension has been historically chronic and incurable resulting in a poor survival rate. Interestingly, new therapeutic treatments are available which have significantly improved prognosis and augmented the survival time, and patients are able to manage the disease for 15 to 20 years or longer. The therapeutic treatment of primary pulmonary hypertension mainly involves calcium channel blockers, anticoagulants, short-acting vasodilators, inhibitors of platelet aggregation, ionotropic agents, corticosteroids or other immunosuppressive agents. Thus, the treatment of pulmonary hypertension is complex and it does not lead to a complete therapeutic cure. Recent studies have shown that sildenafil attenuated pulmonary hypertension by increasing the supply of blood to the lungs [45, 49, 64, 97]. Sildenafil can act by relaxing the arterial wall and by decreasing the pulmonary arterial resistance. Due to the presence of PDE5 in the arterial wall, smooth muscle and lungs, sildenafil acts in these areas and induces vasodilatation. Pfizer submitted an additional registration for sildenafil as an oral therapy for pulmonary arterial hypertension with the FDA, and the drug was approved for this indication in June 2005. The formulation for the pulmonary hypertension therapy was named Revatio, avoiding confusion with Viagra. Revatio is formulated as white, film-coated round tablets equivalent to 20 mg of sildenafil for oral administration.

Sildenafil and pain

Pain is a complex process which involves modulation of both the peripheral and central nervous system [80]. Pain is a self-protective mechanism which forces the body to move away from danger, and afterwards, to rest the injured part, giving the body the chance to heal itself. Pain may be classified according to the origins of pain signal generation. It may be neuropathic, psychogenic, referred, somatic and visceral pain. In acute pain (predominantly nociceptive), visceral, somatic and referred mechanisms play important roles in the pain perception. There have been sufficient studies in recent years indicating that pain perception is no longer a straightforward afferent transmission of pain signal. Pain is perceived as a consequence of the response to electrical (neural) and chemical (hormonal) changes in the body as a result of damage, disease or injury. The signals resulting from any insult, damage or injury are picked up by sensory receptors in nerve endings [125]. Eventually the neurons transmit the signal from the site of injury to the spinal cord, then into the brain where that signal is perceived as pain. Anatomically specific ascending excitatory and descending inhibitory pathways play a role in pain signal transmission. Centralization of the pain signal generators (cephalad relocation in the central nervous system) occurs spontaneously or neural pathways are interrupted, leading to totally unexpected pain syndromes. Scientific evidence shows that acute persistent pain eventually sensitizes wide dynamic neurons in the dorsal horn of the spinal cord, called the wind-up phenomenon. This phenomenon may constitute the basic etiology for chronic pain syndromes. Persistent and excessive pain is harmful to the well being and, therefore, pain needs to be treated as early and as completely as possible. Frequently in non-nociceptive, chronic pain, neuropathic and psychogenic mechanisms prevail, resulting in protracted suffering and disability both physically and mentally [87].

Chronic neuropathic pain, often associated with injury of peripheral or central nerves, has been confirmed to be very difficult to treat therapeutically [87]. Opioids are a major class of analgesics used in management of moderate to severe pain. Nevertheless, treatment with opioids has been found ineffective in alleviating neuropathic pain. Recent studies have shown that NMDA receptor seems to play a major

role in neuropathic pain and in the development of opioid tolerance [41]. Many experiments in both animals and humans have established that NMDA antagonists such as ketamine and dextromethorphan can alleviate neuropathic pain and reverse opioid tolerance. Chronic pain in patients due to spinal cord injury could be reduced by a very low dose of ketamine [33]. Ketamine produces a transient relief from pain associated with burns; however, this has been attributed to its local anesthetic action [86]. Wiesenfeld-Hallin [124] summarized clinical studies which indicated that ketamine could also reduce the need for opiates in the treatment of severe pain. Mathisen et al. [74] showed that chronic neuropathic orofacial pain could be relieved transiently by racemic ketamine and its two stereoisomers, with effective serum concentrations of the three drugs related to their affinity for the NMDA receptor. Major concern with administering NMDA antagonists to treat chronic pain is the impairment in memory and attention [72]. Ketamine can also produce a psychotic state in humans reminiscent of schizophrenic symptoms [66].

Recently sildenafil has been shown to have immense potential for the treatment of pain in animals and humans. Sildenafil produced antinociceptive effect in animal models of pain after local peripheral and systemic administration [3, 8, 61, 84]. Acetylcholine and cholinomimetic agents with predominant muscarinic action are known to increase the concentration of cGMP by activation of the NO signaling pathway in the nociceptive conditions. Patil et al. [85] investigated NO-cGMP-PDE5 pathway in nociceptive conditions in the experimental animals. Acetylcholine or neostigmine (cholinomimetic agent) augmented the peripheral antinociceptive effect of sildenafil. Peripheral accumulation of cGMP may be responsible for antinociceptive effect of sildenafil [62]. There may be a possible interaction between cholinergic agents and PDE5 system in models of nociception [84]. Nearly 50% of diabetes mellitus patients may develop diabetic neuropathy [7, 40]. The treatment of pain in diabetic patients in many ways is unsatisfactory. Anticonvulsants, antidepressants and opioids have become the mainstay in the treatment of chronic neuropathic pain [102]. Sildenafil is a new candidate for a pathogenetically valid treatment in diabetic patients with chronic neuropathic pain [5]. Sildenafil inhibits spinal PDE5 and leads to the accumulation of cGMP that produces intrathecal antinociception. Results of the recent study suggest that cGMP accumulates as a result of PDE5 inhibition and interacts with the cholinergic system to mediate this pain-reducing effect [84].

Sildenafil and multiple sclerosis

Multiple sclerosis is an inflammatory disease of the central nervous system characterized by pathologic changes including demyelination and axonal injury in the brain, spinal cord, and optic nerves. Myelin sheathes surround nerves in the brain and spinal cord. Myelin is lost in multiple areas, leaving plaques or scars called scleroses so named multiple sclerosis. Myelin is the fatty substance that coats and protects these fibers, similar to the way insulation shields electrical wires. Multiple sclerosis is a chronic, potentially debilitating disorder that affects the central nervous system. The central nervous system contains millions of nerve cells joined together to form nerve fibers. Electrical impulses originate in nerve cells and travel along the nerve fibers to and from the brain. Inflammation and injury to the myelin sheath leads to neuronal injury. The result may be multiple areas of scarring (sclerosis) in the central nervous system. Eventually, this scaring or sclerosis can slow or block the nerve signals that control muscle coordination, strength, sensation and vision. Multiple sclerosis affects approximately 400,000 individuals in the United States and 2.5 million worldwide, with a typical onset during the productive years between the ages of 20 and 50. Multiple sclerosis is more predominant in women. Multiple sclerosis patients experience their first symptoms between the ages of 20 and 40. Symptoms of multiple sclerosis vary widely, depending on the location of affected nerve fibers. Signs and symptoms of multiple sclerosis may include fatigue, numbness or weakness of limbs, partial or complete loss of vision, double vision or blurring of vision, tingling or pain in numb areas of the body, electric-shock sensations that occur with certain head movements, tremor, lack of coordination or unsteady gait and dizziness. Some patients may also develop slurred speech, muscle stiffness or spasticity, paralysis, or problems with bladder, bowel or sexual function. Mental changes such as forgetfulness or difficulties with concentration also can occur. Beta interferons, glatiramer (Copax-

one), mitoxantrone (Novantrone), corticosteroids, muscle relaxants and medications to reduce fatigue are the current therapeutic strategies used in multiple sclerosis.

Multiple sclerosis leads to memory impairment through disturbances in the cortical and subcortical pathways as a consequence of demyelination and axonal transection. Neurologists employ magnetic resonance imaging (MRI) to track the effect of drug therapy on the development or lack of new lesions [43]. Sildenafil has been shown to protect multiple sclerosis patients from neurodegeneration through increased gray matter perfusion in the brain [73]. Functional MRI taken after oral administration of sildenafil has shown significant increase in gray matter perfusion in multiple sclerosis patients. Sildenafil has been shown to enhance neurogenesis suggesting its role in neuroprotection in multiple sclerosis.

Sildenafil and neurogenesis

The old hypothesis regarding neurogenesis, or the birth of new neuronal cells, suggested that neurogenesis could occur only in developing organisms. However, recent scientific studies have demonstrated that neurogenesis occurs continuously into and throughout adult life in both vertebrate and invertebrate organisms [48, 116]. Neurogenesis is significant in the hippocampus of mammals [1, 36, 120], song control nuclei of birds [2] and the olfactory pathway of rodents [71], insects [22] and crustaceans [50]. Neurogenesis occurs in adult forebrain regions of the subventricular zone and the dentate gyrus in various species [117]. Ongoing neurogenesis is thought to be an important mechanism underlying neuronal plasticity, enabling organisms to adapt to environmental changes and influencing learning and memory throughout life [103, 106]. Neurogenesis, essential for synaptic plasticity and formation of memory, generally declines with age and is associated with neurodegenerative diseases [89, 123]. Recently, numerous factors that regulate neurogenesis have been identified. Physical activity and environmental conditions are significant aspects that have been known to affect proliferation and survival of neurons in vertebrates as well as invertebrates. Crayfish in an enriched environment had improved neurogenesis and neuronal survival compared to siblings in an "impoverished" environment [70]. Hormones, such as testosterone and adrenal steroids, have also been found to influence the rate of neurogenesis in vertebrates and invertebrates [21]. Serotonin is known to play a key role in neurogenesis in a variety of organisms. In lobsters, serotonin depletion significantly decreased the proliferation and survival of olfactory projection neurons and local interneurons. Interestingly, neurogenesis follows a circadian rhythm in the juvenile lobster. Even though new neurons are generated regularly throughout the day, extensively more neurons were generated in the evening or night, the most active time for lobsters. Studies in animals showed that both serotonin reuptake inhibitors and the antiepileptic drug phenytoin (dilantin) blocked the effects of stress on the hippocampus.

Preclinical studies indicate that stress is associated with changes in structure of the hippocampus. The hippocampus is the area of the brain which plays a critical role in memory and neurogenesis. Imaging studies measuring magnetic resonance have found a smaller volume of the hippocampus in patients with post-traumatic stress disorder related to both combat and childhood abuse. These patients were also found to have deficits in memory by neuropsychological testing. Functional imaging studies using positron emission tomography found decreased hippocampal activation with memory tasks. However, paroxetine significantly increased the hippocampal volume leading to enhanced memory function. Like paroxetine, phenytoin was also effective in post-traumatic stress disorder in increasing hippocampal volume but without significant change in memory [19].

Sildenafil also enhances functional recovery and neurogenesis after stroke in rats [126–128]. Sildenafil has been shown to increase cGMP levels in the brain, induce neurogenesis and reduce neurological deficits in rats after stroke [39]. Neuronal growth is decreased with age mainly due to lowered production of cGMP and stroke reduces the number of functional neurons in the brain. Prenatal brain development is influenced by neuronal nitric oxide synthase and suggests a role for cGMP in both neurogenesis and synaptogenesis [23]. Production of NO and cGMP is reduced with age [115] suggesting that decreased cGMP levels could contribute to age-related decrements in neurogenesis. Therefore, studies on aged animals have important clinical implications for stroke treatment. Stroke remains as a major cause of death and disability in aged population [82]. Sildenafil promotes cell prolif-

eration in neurospheres isolated from the subventricular zone of adult rat [122]. PDE5 is expressed in neurospheres [11, 122]. Sildenafil significantly increased cGMP levels and neurogenesis in neurospheres. Sildenafil also significantly phosphorylated protein kinase B (Akt) in neurospheres. Phosphorylated Akt has been associated with an increase in phosphorylation of glycogen synthase kinase 3 (GSK-3), a downstream target of Akt. This was confirmed in a study with PI3-K/Akt inhibitor, LY 294002. Co-incubation of LY 294002 with neurospheres abolished the phosphorylation. This study suggests that Sildenafil-enhanced neurogenesis likely occurs through activation of the PI3-K/Akt/GSK-3 pathway. Sildenafil increases the cGMP levels and enhances neurogenesis in cell culture studies [122]. Suzuki et al. [114], have shown that glutamate can significantly increase the proliferation rates of human neural progenitor cells. Therefore, it is clear that glutamate-NO-cGMP pathway plays a role in sildenafil-mediated neurogenesis. The use of specific PDE5 inhibitors such as sildenafil may, therefore, offer an innovative approach for improvement of brain function in the aged population.

Sildenafil and memory enhancement

Alzheimer's disease is a progressive neurodegenerative disorder that is mainly characterized by cognitive impairment. An estimated 4 million people, most of them elderly, have Alzheimer's disease in the United States, affecting 30–50% of individuals aged 85 and older [37]. The specific cause of Alzheimer's disease is unknown, but genetic abnormalities appear to play a role and neuroinflammation is now recognized as a prominent feature in Alzheimer's pathology [75]. Therapeutic treatment consists of alleviating symptoms, providing long term care at a minimal cost with fewer adverse effects. Progressive neurodegeneration results in chronic cognitive decline culminating in memory loss and motoneuronal dysfunctions. Alzheimer's disease patients find difficulty reasoning, making judgments, communicating and carrying out daily activities. With the progression of Alzheimer's, patients may also experience changes in personality, behavior and life style, such as anxiety, suspiciousness or agitation, as well as delusions or hallucinations [56].

Prevalence of Alzheimer's disease is increasing in trend, particularly in economically developed countries due to many reasons including increase in life expectancy and change in food habitts. Huge investments are currently made for the therapeutic intervention and cure of Alzheimer's disease. The cholinergic and glutamatergic neurotransmitter systems, which share a close functional relationship, may play a role in the pathogenesis of Alzheimer's disease. Acetylcholinesterase inhibitors (AChEI) are effective for the treatment of mild to moderate Alzheimer's disease. Currently the three cholinesterase inhibitors donepezil, rivastigmine, and galantamine are widely recommended for clinical use [14, 63]. Galantamine is of particular interest because it has a dual mechanism of action: it is postulated to be both an AChEI and an allosteric modulator of nicotinic receptors. Memantine, an N-methyl-D-aspartate (NMDA) inhibitor, has been approved for the therapeutic use in moderate to severe Alzheimer's disease [25, 44]. Modulation of NMDA and nicotinic receptors by memantine and galantamine may provide an optimal combination therapy for Alzheimer's disease. However, there is no perfect drug currently available for the treatment. Recent studies have shown that PDE5 inhibitors can counteract deficits in long-term memory caused by pharmacological agents or aging [27, 29, 34, 35]. Therefore, targeting PDE5 with a selective inhibitor like sildenafil may offer a novel therapy aimed at slowing progression, prevention and, eventually, therapy of Alzheimer's disease. Event-related brain potentials recorded following sildenafil administration suggest an enhanced ability in young men to focus attention on auditory stimuli [107]. This finding is significant as the first attempt to study the cognitive effects of sildenafil in humans using electrophysiological techniques. Animal studies have shown sildenafil to enhance memory [10, 27, 28, 30, 34, 90–93, 101, 111]. Administration of sildenafil directly into the hippocampus after the first trail in object recognition task, improved memory in mice [77, 101] and enhanced the processes of consolidation of object information [93]. Similarly, sildenafil has been shown to attenuate memory impairment induced by nitric oxide synthase inhibition [30], hyperammonemia [34, 35] and blockage of muscarinic cholinergic receptors [27]. Sildenafil administration improved the cognitive performance in diabetic conditions and electroconvulsive shock-induced animal models [83].

There are several theories proposed to explain the memory enhancement by phosphodiesterase inhibition. PDE5 inhibition causes vasodilatation, probably through cGMP, in rats [31, 32]. Thus, one of the suggested mechanisms is memory improvement through increased blood flow and consequent glucose metabolism in the brain [93]. The NO-cGMP-cGK pathway is involved in learning-related forms of synaptic plasticity [92]. Studies have revealed a variety of molecular mechanisms, including retrograde signaling and activation of presynaptic PKG and calmodulin-dependent protein kinase II (CaM KII) for the expression of long-term potentiation (LTP). LTP in the associational/commissural pathway requires NMDA receptor activation, postsynaptic depolarization, a rise in postsynaptic Ca^{2+}, and insertion of postsynaptic AMPA-type glutamate receptors [108]. Induction of LTP is blocked by inhibition of nitric oxide synthase [16]. Evidence from this study and other reviews implicates NO as a retrograde messenger in memory mechanism [28]. Though LTP is considered as a postsynaptic event, retrograde signaling of NO followed by cGMP-stimulated release of glutamate is suggested as a presynaptic mechanism [6, 16, 53]. Cascade of events related to memory enhancement by sildenafil is presented in Figure 2. A unifying hypothesis intended to explain the sildenafil-mediated memory enhancement proposes that accumulation of cGMP initiates a complex cascade. Presynaptic PDE5 inhibition increases the cGMP level and triggers the release of glutamate and subsequent NMDA receptor activation. Postsynaptic inhibition of PDE5 increases protein synthesis and synaptogenesis. Increased activity of cGMP-coupled ion channels may lead to early consolidation of information into memory [65, 107]. Electrophysiological experiments with long-term potentiation revealed that cGMP had to be kept high but below a certain threshold to reach the peak capacity to learn [34].

Bernabeu et al., found that administration of cAMP into the hippocampus enhanced passive avoidance learning, suggesting cAMP involvement in later stages of memory consolidation processes [12]. Direct administration of cGMP into the hippocampus improved object memory in rats whereas there was no improvement by cAMP. Cyclic GMP-regulated processes in the hippocampus play a significant role in the early stages of memory consolidation and cAMP

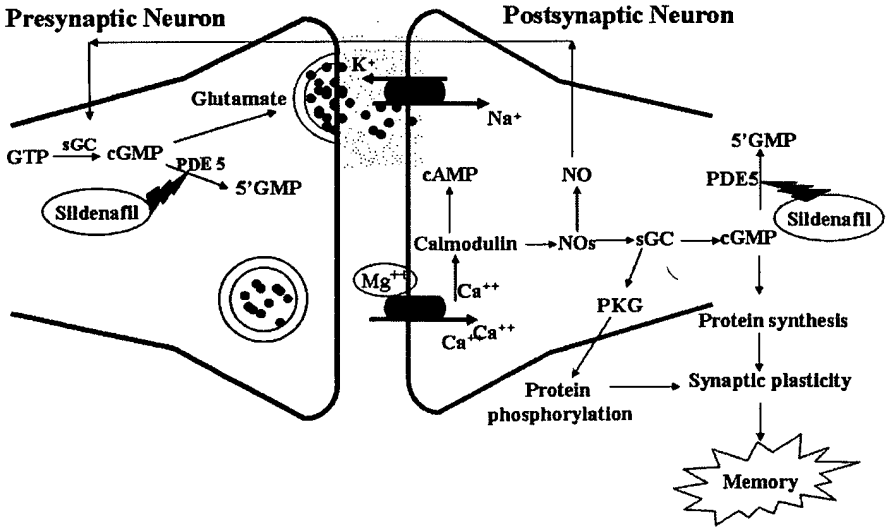

Fig. 2. Schematic diagram for cascade of events related to memory enhancement by sildenafil: cGMP-glutamate-calmodulin-sGC-PKG pathway is activated during NMDA receptor-dependent long term potentiation (LTP) in the CA1 region of the hippocampus. Activation of ionotropic (mainly NMDA) glutamate receptors leads to increased intracellular calcium that binds to calmodulin and activates neuronal nitric oxide synthase, leading to increased production of nitric oxide (NO). NO in turn activates sGC resulting in increased formation of cyclic guanosine monophosphate (cGMP). Increased formation of cGMP leads to protein synthesis, synaptogenesis and memory enhancement. Sildenafil acts on the phosphodiesterase-5 (PDE5) and increases the availability of cGMP and with low release of NO may enhance the memory

signaling pathways are occupied in the late post-training memory processing of inhibitory avoidance learning [10, 13]. LTP is mainly a postsynaptic event. However, LTP is expressed by increasing the presynaptic release of glutamate through the GC/cGMP/PKG pathway [23, 27, 28, 65]. Glutamate-NO-cGMP pathway modulates important cerebral processes such as intercellular communication, the circadian rhythms and LTP [15, 18]. Sildenafil may also reduce the cognitive deficits associated with aging and be used for treating age-related neurodegeneration. Production of NO and cGMP is reduced with age and to some extent contributes to age-related memory decline [115]. Sildenafil, a specific inhibitor of cGMP degrading enzyme, therefore, offers a new strategy for memory improvement and a novel therapy for Alzheimer's disease in the future.

Adverse effects of sildenafil

Few incidences of transient ischemic attack following sildenafil medication have been published [57, 78, 81]. In many cases, patients acquired risk factors sufficient to develop transient ischemic attack and sildenafil might have provoked the incidence [78, 96]. Although a definitive cause has not been determined, transient ischemic attack is thought to occur through reduced blood pressure. Sildenafil may cause venodilation, brief arrhythmia and cerebral arterial vasculature in patients with sympathetic cereberovascular disease. This leads to lowering of blood pressure across diseased artery resulting in transient ischemic attack and followed by stroke. Sildenafil may also cause increase in sympathetic activity and subsequently stroke [88]. A small number of patients have reported developing seizures subsequent to taking sildenafil and this is mostly among patients with other complications like hypertension [46]. Due to inhibition of PDE5, sildenafil causes accumulation of cGMP which triggers the release of glutamate [98]. The increased release of glutamate may cause seizures in susceptible individuals [51]. Few cases of amnesia have been reported after sildenafil medication [42, 105]. Susceptible people will develop ischemia in hippocampal region leading to transient global amnesia [69]. Migraine is a risk factor for transient global amnesia. Nonarteritic anterior ischemic optic neuropathy (NAION) has been reported rarely in men following sildenafil medication [47]. Hayreh [54] has reviewed the oral treatment of erectile dysfunction and non-arteritic anterior ischemic optic neuropathy. Blue tinge to vision, increased brightness of lights, and blurry vision are reported in many occasions particularly at higher dose of sildenafil [60]. Sildenafil has a relatively low IC_{50} for PDE5 that was found in rod and cone photoreceptor cells of retina [59]. Prevalence of impaired vision caused by sildenafil could be explained by the elevated cGMP in retina [68]. Cyclic nucleotide-gated channels are found in the membrane of cone outer segments and at the synaptic terminal [99, 104]. Inhibition of NO synthesis may induce anxiety [38]. Sildenafil in many cases helps men fight anxiety through treating the erectile dysfunction. Depression, anxiety and psychosocial problems are high in men with erectile dysfunction and were ameliorated by sildenafil [26, 76, 79, 109, 113]. However, animal studies have shown an anxiogenic effect of sildenafil [58, 67, 121]. A small number of men also reported developing anxiety after taking sildenafil. Sildenafil may increase the cGMP levels and consequently reduce the NO release in the brain through a negative feedback mechanism. Sildenafil may also have other common side effects such as headache, upset stomach, diarrhea, chest pain, dizziness or lightheadedness, fainting, flushing, itching or burning during urination, rash, stuffy nose, prolonged and painful erection. Widespread clinical use of sildenafil and review from consumers around the world reveal that problems with acute side effects and health risks are minimal for the majority of patients [20, 28, 59].

Outlook

This review summarizes the pharmacological effect of sildenafil in pulmonary hypertension and central nervous system and also discusses the adverse effects. Sildenafil is chemically characterized as a pyrazolopyrimidine derivative and biochemically as a PDE5 inhibitor. With regard to its biological effects, evidence from clinical trials and experimental studies suggest that sildenafil exhibits a plethora of pharmacological actions. Although still in the preclinical phase, promising results have been reported for sildenafil from studies in stroke. While maintaining excellent safety

and tolerability profile in the treatment of erectile dysfunction, sildenafil presents a prolonged benefit in the central nervous system, pulmonary hypertension, and pain. Aspects of neurogenesis, memory enhancement, neuroprotection and antinociception are encouraging and need further investigation. Basically, sildenafil acts through cGMP, however, there are many lines of its action for investigation.

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